The use of 3-aminobenzamide as a probe for the function of ADP-ribosylation in vivo.

Purnell, Michael R.

Award date:
1981

Awarding institution:
University of Bath

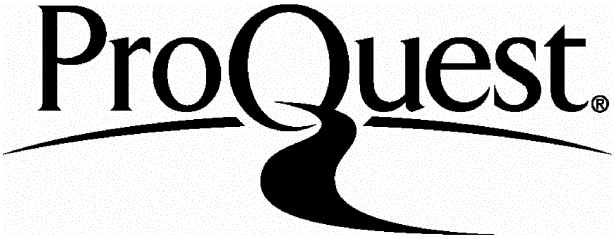
THE USE OF 3-AMINOBENZAMIDE AS A PROBE FOR THE FUNCTION OF ADP-RIBOSYLATION IN VIVO

submitted by Michael R. Purnell
for the degree of Ph.D. of the
University of Bath

1981

M R Purnell

M. R. Purnell



ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr W. J. D. Whish, for his constant help, interest and encouragement throughout this work. My thanks also go to various members of the Biochemistry Department, University of Bath, especially Dr R. S. Eisenthal and Dr P. R. Stone, for their stimulating discussions.

I would finally like to thank the Science Research Council and Miles Laboratories, Stoke Poges, U.K. for their financial support.

SUMMARY

The synthesis of various benzamides is described. These, and various other compounds, were screened for their ability to inhibit the nuclear enzyme, poly(ADP-ribose) synthetase. Benzamide and various analogues substituted in the 3-position were more potent inhibitors than any described previously. 3-Aminobenzamide and 3-methoxybenzamide inhibited the enzyme competitively with respect to its substrate, NAD.

3-Aminobenzamide was shown to inhibit ADP-ribosylation in permeabilized L1210 cells but no other enzymes involved in NAD metabolism. It was demonstrated that 3-aminobenzamide entered L1210 cells. No effect on cell proliferation was observed at concentrations up to 5mM. At concentrations above this, a decrease in the rate of division was seen. Radioactive thymidine and uridine incorporation into acid-insoluble material was unaffected by 2mM 3-aminobenzamide. [^{3}H]-Adenosine incorporation was inhibited and [^{3}H]-leucine incorporation was transiently stimulated. NAD levels in L1210 cells increased upon 3-aminobenzamide treatment compared to controls.

The suitability of 3-aminobenzamide as a probe *in vivo* and the approach of using inhibitors of poly(ADP-ribose) synthetase *in vivo* are discussed. Regulatory functions previously ascribed to ADP-ribosylation of nuclear proteins are discussed in view of the present findings.

TABLE OF CONTENTS

1. INTRODUCTION 1

1.1 ADP-ribosylation in general 1

1.2 ADP-ribosylation of nuclear proteins 3

1.2.1 The structure of mono- and poly(ADP-ribose)-protein conjugates 3

1.2.2 Identification of proteins modified *in vitro* 6

1.2.3 Identification of protein acceptors *in vivo* and quantification of mono and poly(ADP-ribose) 9

1.2.4 Synthesis of poly(ADP-ribose) 14

1.2.5 Degradation of poly(ADP-ribose) 23

1.3 25

1.3.1 Biological role of ADP-ribosylation 25

1.3.2 DNA synthesis and cellular proliferation 28

1.3.3 DNA transcription 33

1.3.4 DNA repair 36

1.3.5 Cell cycle 40

1.3.6 Cellular differentiation 43

1.3.7 Other functions 46

1.3.8 Possible mechanisms by which ADP-ribosylation may affect cellular processes 48

1.4 Rationale for the present work 51

2. ORGANIC SYNTHESES 54

2.1 Purification of 3-aminobenzamide 54

2.2 Synthesis of 3-aminobenzamide 55

2.3 Synthesis of 3-bromobenzamide 57

2.4 Synthesis of 3-hydroxybenzamide 58

2.5 Synthesis of 3-acetylaminobenzamide 60

2.6 Synthesis of 3-succinylaminobenzamide 60

2.7 Synthesis of 3-nitrobenzamidomethane 61

2.8 Synthesis of 3-nitrobenzamidoethane 62

2.9 Synthesis of 3-aminobenzamidomethane 62

3. SCREENING OF POTENTIAL INHIBITORS OF POLY(ADP-RIBOSE) SYNTHETASE 64

3.1 Introduction 64

3.2 Methods 65

3.2.1 Isolation of pig thymus nuclei and extraction of poly(ADP-ribose) synthetase with 0.5M NaCl 65

3.2.2 Poly(ADP-ribose) synthetase assay 66

3.2.3 Effect of 3-aminobenzamide on the stability of the ADP-ribose - protein linkage 66

3.3 Results and Discussion 67

3.3.1 ADP-ribosylation and the effect of benzamides 67

3.3.2 The effect of acetophenones on ADP-ribosylation 72

3.3.3 The effect of benzoic acids on ADP-ribosylation 73

3.3.4 The effect of other compounds on ADP-ribosylation 75

4. THE EFFECT OF 3-AMINOBENZAMIDE ON L1210 CELLS 77

4.1 Introduction 77

4.2 Methods 78

4.2.1 Poly(ADP-ribose) synthetase assay 78

4.2.2 NAD glycohydrolase 80

4.2.3 NMN pyrophosphorylase 82

4.2.4 NAD pyrophosphorylase 82

4.2.5 Entry of 3-aminobenzamide into L1210 cells and the metabolism of 3-aminobenzamide 83

4.2.6 Effect of 3-aminobenzamide on cell proliferation 84

4.2.7 Effect of 3-aminobenzamide on the incorporation of precursors into macromolecules 84

4.2.8 NAD levels in L1210 cells 85

4.2.9 Poly(ADP-ribose) synthetase activity in permeabilized cells following treatment with 3-aminobenzamide *in vivo* 85

4.3 Results and Discussion 86
4.3.1 Poly(ADP-ribose) synthetase 86
4.3.2 NAD glycohydrolase 86
4.3.3 NMN pyrophosphorylase 89
4.3.4 NAD pyrophosphorylase 90
4.3.5 Entry of 3-aminobenzamide into the cell and metabolism 90
4.3.6 The effect of 3-aminobenzamide on cell proliferation 92
4.3.7 Incorporation of radioactive precursors into acid-insoluble material 93
4.3.8 The effect of 3-aminobenzamide on NAD levels in L1210 cells 95
4.3.9 Poly(ADP-ribose) synthetase activity following 3-aminobenzamide treatment 96
4.4 General discussion 97

5. CONCLUSIONS 106

6. REFERENCES 107

7. APPENDIX - ADP-ribosylation of thiol-containing proteins 125

8. PUBLICATIONS

SECTION 1

INTRODUCTION

1. INTRODUCTION

ADP-ribosylation is an enzyme-catalysed post-synthetic modification of proteins by the ADP-ribose moiety of NAD. It is known to be responsible for the inhibition of protein synthesis by both diptheria and P.aeroginosa toxins. The catalytic subunits of both these toxins inactivate elongation factor Tu (1). The amino acid modified by diptheria toxin has been recently identified as 2-[3-(carboxyamido) -3-(trimethylammonio) propyl] histidine (2). Both cholera toxin and E.coli heat-labile enterotoxin exert their effects by activating adenyl cyclase (1,3). This activation is the consequence of the ADP-ribosylation of a regulatory protein, which has been identified as the GTP-binding protein (4,5). The amino acid acceptor for both toxins has been identified as arginine (6).

ADP-ribosylation of RNA polymerase has been observed following the infection of E.coli by T4 (7,8,9). The ADP-ribosylation takes place in two distinct phases. Immediately following infection the enzyme is ADP-ribosylated by a phage protein. This has been termed alteration. After transcription of the phage gene, RNA polymerase is modified again, presumably by a protein encoded in the T4 DNA. This ADP-ribosylation has been termed modification. In both cases, the α-subunits of RNA polymerase are ADP-ribosylated at the same site. The modified enzyme transcribes the phage DNA preferentially. Another coliphage, N4, has also been reported to contain an intrinsic ADP-ribosyl transferase activity (10).

The first demonstration of ADP-ribosylation of proteins was that which was found to occur in the nuclei of eukarotic cells (11,12,13). This followed the observation that nicotinamide mononucletide stimulated the incorporation of [^{3}H]-ATP into acid-insoluble material by hen liver nuclei (14). The enzyme responsible for this modification is called poly(ADP-ribose) synthetase or polymerase. Its name is derived from the fact that unlike the ADP-ribosyl transferases mentioned above, poly(ADP-ribose) synthetase is able to make a protein bound homopolymer of ADP-ribose, poly(ADP-ribose).

ADP-ribosylation, in general, and poly(ADP-ribosyl)ation in particular have been extensively reviewed principally by Sugimura (15) Hilz & Stone (1) and Hayaishi & Ueda (16). This introduction will be concerned exclusively with eukaryotic ADP-ribosylation (unless otherwise stated, ADP-ribosylation refers to modification by either mono or poly (ADP-ribose)) and will concentrate on data which has emerged since the above reviews were published.

1.2. ADP-ribosylation of Nuclear Proteins

1.2.1 The structure of mono- and poly(ADP-ribose)-protein conjugates

ADP-ribose is attached to proteins through the free ribose molecule by two distinct types of bonds and the observation has been made that following incubation of isolated rat liver nuclei with NAD, radioactively labelled in the adenine moiety, all radioactivity is removed by dilute alkali but only 60% is removed by neutral hydroxylamine (17). Endogenous mono-ADP-ribose residues linked to proteins isolated from E.A.T. cells and rat liver have also been reported to possess similar labilities (18) although the relative proportions of each type of linkage are different (see section 2.3) in the two tissues. More recently, it has been suggested that a third, alkali-resistant bond may exist *in vivo* (19). It should be mentioned here that poly(ADP-ribose) is stable in alkali (15,20).

Proposals as to the nature of the linkage to protein vary from a carboxylic acid ester (21,22), a Schiff base with lysine (23) or a phosphodiesterase linkage with phosphoserine (24,25). The carboxylic acid ester was proposed to account for the lability towards hydroxylamine. It has since been reported that glutamic acid residues at position 2 are ADP-ribosylated in both histones H1 and H2B (26,27). Hayaishi *et al* (28) have also reported ADP-ribosylation at these positions and, in addition, at positions 4 and 116 in H1. More recently, the same group

reported the COOH terminal was also modified (29). Both groups used isolated nuclei from rat liver.

The chemical structure of poly(ADP-ribose) was first elucidated by Doly (11). The anomeric carbon of one ADP-ribose was found to be attached to the adenosine moiety of the next via a 1"-2' glycosidic linkage. Subsequent NMR analyses of either pure poly (ADP-ribose) (30) or its degradation product, 2'-(5"-phosphoribosyl)-5' AMP or PR-AMP (30-32), have identified the linkage as α(1"-2') - see Figure 1.

Recent studies on the chain length of poly(ADP-ribose) formed in isolated nuclei show the product may have as many as 65 ADP-ribose residues. The earliest method of determining the chain length was by incubating isolated nuclei with radioactively labelled NAD, digesting the product with snake venom phosphodiesterase, resolution of 5'AMP and PR-AMP by paper chromatography and analysis of the radioactivity associated with each compound. The ratio of the total radioactivity (AMP and PRAMP) to the radioactivity in AMP gives an average chain length (22). The first method of resolving poly(ADP-ribose) of differing chain lengths was that of Sugimura *et al* (33). Isolated nuclei labelled with [^{14}C]-NAD were digested with pronase and the poly(ADP-ribose) and nuclei acids were precipitated with ethanol. The pellet was resuspended and subjected to hydroxylapatite column chromatography; elution was effected with increasing concentrations of phosphate buffer. In addition to separation of nucleic acids from

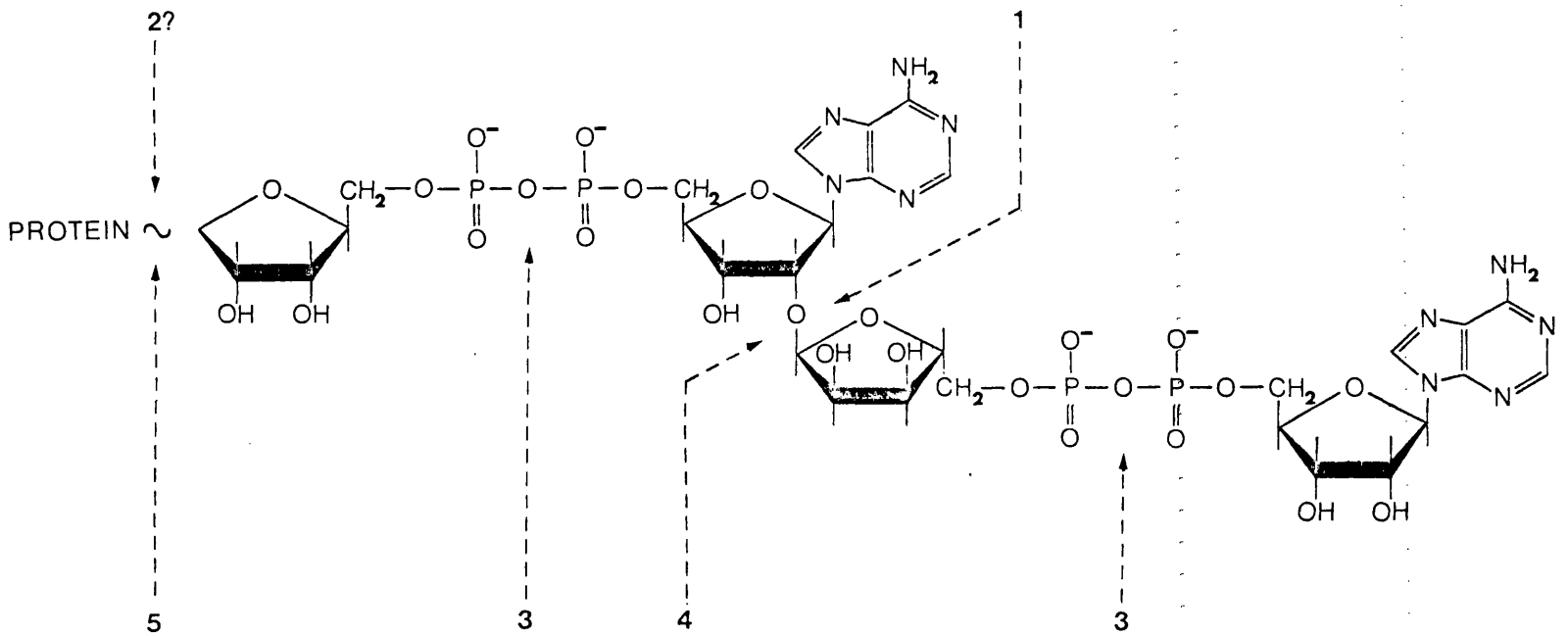


Figure 1 Structure and enzymology of poly(ADP-ribose).

1. Elongation 2. Initiation 3. Phosphodiesterase
4. Poly(ADP-ribose) glycohydrolase
5. ADP-ribose - protein hydrolase

poly(ADP-ribose), they observed a linear relationship between chain length and concentration of phosphate. The same group later analysed each of the observed peaks by gel electrophoresis and found each peak comprised of two subfractions differing in both chain length and terminal structure (34). They suggest the latter may be due to partial degradation by hydrolytic enzymes.

Adamietz et al (35) used polyacrylamide gel electrophoresis to separate poly(ADP-ribose) molecules of differing chain lengths after alkaline degestion of labelled isolated nuclei. Discrete bands corresponding to poly(ADP-ribose) of up to 33 residues in length were detected by fluorography. They were able to show quantitative differences in the chain length pattern from nuclei isolated from different tissues by determining the radioactivity in each band.

A similar protocol was employed by Tanaka et al (36) to resolve various fractions of poly(ADP-ribose) after hydroxylapatite column chromatography. They were able to show the presence of at least 65 discrete bands. After analysing the chain length of each band by the method of Nishizuka et al (22), they found an increasing chain length up to (ADP-ribose) 30. Bands with slower mobilities, however, showed no increased chain length by the phosphodiesterase method. They suggest this could be due to the presence of a branched polymer having more than one AMP terminus. Recently, Miwa et al (37,38) have isolated and

identified a branched structure whereby additional ADP-ribose molecules are attached to poly(ADP-ribose) via a 1"'-2" glycosidic linkage with the ribose to which the nicotinamide was originally attached.

Farzaneh & Pearson (39) used the hydroxylapatite column chromatography method to determine the chain length of poly(ADP-ribose) during development of Xenopus laevis. In all cases they found this method gave larger estimates than the phosphodiesterase method of Nishizuka et al (22). They proposed that [^{3}H]-NAD was incorporated in vitro onto pre-existing chains synthesised in vivo. The same group have recently reported a similar result after analysing the reaction product from BHK cells (40).

1.2.2 Identification of proteins modified in vitro

The general method for determining the number of protein acceptors has been to isolate nuclei and then incubate with radioactive NAD. After electrophoresis and autoradiography, the radioactive regions can be compared with gel stained for protein. Using this method, a large number of proteins have been shown to be modified in a wide variety of tissues (41-43). Both histones and non-histone proteins are ADP-ribosylated although the relative proportion of label in each fraction varies between different tissues (see review by Hilz & Stone (1)). Recently, a powerful new technique was reported for the specific isolation ADP-ribosylated protein from a reaction mixture (44). The chromatographic method employs the

formation of a complex between the cis diols of mono and poly(ADP-ribose) and immobilized aminophenyl boronic acid. The authors have shown that in rat liver nuclei, histones H1, H2A, H2B and the HMG proteins were modified (28). More recently, the same group also reported protein A24 was ADP-ribosylated (45).

The ease with which relatively pure histones can be obtained from nuclei e.g. inorganic acid extraction and Bio - Rex 70 chromatography, has meant that most workers have concentrated their attention on these proteins (see [1]). From the data obtained, the major histone acceptor has been reported to be H2B in rat or mouse liver (44) wheat embryos (46) and cultured nicotinia tabaccum cells (47), whereas in HeLa cell nuclei (43), rat brain or MS cell nuclei (41), the major acceptor was H1. Tanuma et al (48) found that the bulk of the radioactivity migrated slower than H1. Stone et al (49) on finding a similar result characterized this material and found it consisted of two H1 molecules joined by a single poly(ADP-ribose) molecule with an average chain length of 15. Similar results have also been reported by Smulson's group in HeLa cells (50), Hilz's group in HeLa cells (51) and by Kidwell's group in HBL-100 cells and lactating rat mammary gland (52).

Recent findings by Adamietz et al (53) cast doubt as to the validity of identifying protein acceptors by mobility on gel electrophoresis. They used a boronate column to

isolate [^{3}H]-ADP-ribosylated proteins from E.A.T. cell nuclei. The proteins were then separated into a histone fraction and a non-histone fraction by Bio-Rex 70 chromotography. SDS gel electrophoresis and protein staining of the histone fraction revealed two major bands, one slightly ahead of H2B and the other ahead of H4 with two minor bands between these two. If, however, this fraction was treated with alkali to remove the ADP-ribose, the major acceptors were identified as H2B and H4 and to a lesser extent H3 and H2A. More striking results were observed after electrophoresis of the non-histone fraction. Besides major bands with molecular weights ranging from 31,000 to above 120,000, they observed a series of fine bands with molecular weights ranging from 12,000 to 30,000. When treated with alkali, these disappeared and the major stained bands corresponded to the core histones (H2A,B,3 & 4). Thus heavily modified histones not only behave differently as regards their mobility on SDS gel electrophoresis but also on ion exchange chromatography.

A second approach to determine whether a specific protein is an acceptor has been to add the purified protein to a mixture of purified poly(ADP-ribose) synthetase, DNA and radioactive NAD. In this manner a Ca^{2+}, Mg^{2+} dependent endonuclease (54) and poly(ADP-ribose)synthetase (55) and RNA polymerase B(56) have been shown to act as acceptors. When Okayama *et al* (57) attempted to show ADP-ribosylation of histones they could not detect any radioactivity migrating with protein . The bulk of the radioactivity migrated with

an Rf of 0.58. This has since been shown to be NAD because the material was present when the enzyme was omitted or when NAD alone was electrophoresed (58). Recently, however, three groups have demonstrated ADP-ribosylation of histones using this method (for details see section 2.4).

1.2.3 Identification of protein acceptors in vivo and quantification of mono and poly (ADP-ribose)

In contrast to modification *in vitro*, very little research to date has been directed at the identification of proteins ADP-ribosylated *in vivo*. The major problem is the lack of a specific precursor for radioisotopic studies. The components of ADP-ribose, viz adenine, ribose and phosphate, are incorporated into compounds such as nucleic acids, glycoproteins and phosphoproteins. Thus for tracer studies these contaminents must be removed before analysis can begin. For this reason, the most studied proteins have been the histones because of the relative ease with which they can be purified. ADP-ribosylation of histones was first demonstrated by Hayaishi's group in 1975 (59). After the injection of rats with [^{14}C]-ribose, the livers were excised and nuclei isolated. The histones were extracted with 0.25M HCl and then purified by CM-cellulose chromatography. The radioactivity eluted with H1, H2 and H3. Ord and Stocken (60) used a similar method to show H1 was modified. In addition a small peptide and protein P1 were ADP-ribosylated. Adamietz *et al* (51) compared ADP-ribosylation of H1 *in vitro* and *in vivo* in

HeLa cells. The H1 was obtained by perchloric acid extraction of [^{3}H]-adenosine labelled cells followed by Bio-Rex 70 chromatography. After snake venom phosphodiesterase digestion, the majority of radioactive material migrated as AMP with the remainder migrating as PR-AMP. Thus in HeLa cells, H1 was modified predominantly with mono-(ADP-ribose) but also with oligomeric material. H1 was mainly assoiated with poly(ADP-ribose) when nuclei were labelled *in vitro*. Thus, ADP-ribosylation of H1 *in vivo* is quite different from that observed in isolated nuclei. Modification of histones by poly(ADP-ribose) (greater than 4 units long) was studied by means of an anti-poly(ADP-ribose) antibody (see below) (61). Less than 1% of the total poly(ADP-ribose) in rat liver was associated with histones. This histone associated material was evenly distributed between H1, H2A, H2B and H3.

The first attempt to quantify levels of poly(ADP-ribose) *in vivo* was the isotope dilution method of Stone *et al* (62). [^{3}H]-poly(ADP-ribose) of known specific activity (synthesized *in vitro*) was added to a homogenate of rat liver. This was then treated with alkali and hydroxylamine to release endogenous poly(ADP-ribose) from proteins. After partial purification by ion exchange, the material was digested with phosphodiesterase and PR-AMP was purified by chromatography. The final step consisted of conversion to ribosyl-adenosine and purification to constant specific radioactivity. From this and the known starting specific activity the amount of endogenous poly(ADP-ribose) could be

calculated. Values of 5.59 nmoles/mg DNA were obtained for adult rat liver and 6.32 nmoles/mg DNA for neonatal.

Two groups have used fluorimetric assays for adenosine to quantify poly(ADP-ribose) levels *in vivo*. Mandel's group (63) treated isolated nuclei from rat liver with alkali to release the poly(ADP-ribose) and used a four step procedure to purify it. After enzymic hydrolysis to either PR-AMP or ADP-ribose, they reacted the product with glyoxal and determined the levels spectrofluorometrically. The value for adult rat liver obtained was 3.35 nmoles/mg DNA. The second method (64,65) was used to estimate levels in 3T3 cells. After acid precipitation of the cells, the pellet was resuspended in 6M guanidine HCl and applied to a boronate column (see previous section). After washing, the poly(ADP-ribose) was digested to nucleosides with phosphodiesterase and phosphatase. The nucleosides were then eluted and reacted with chloroacetaldehyde to give the etheno-adenosine derivatives which were separated by HPLC. The value obtained was 5 pmoles of ribosyl adenosine/10^8 cells.

Antibodies against poly(ADP-ribose) have been used as the basis for determining levels *in vivo* radioimmunologically. The first report of antibodies raised against poly(ADP-ribose) was from Sugimura's group (66). The final preparation was highly specific and could detect levels of 5μg in the assay. The authors later reported levels in pig thymus to be 0.2μg/mg DNA (67). Kidwell & Mage (68)

used similar antibodies to study the levels of poly(ADP-ribose) during the cell cycle of HeLa cells. The maximum level observed was 83 ng/mg DNA. Kun's group raised antibodies against poly(ADP-ribose)$_{40}$ and estimated levels in various tissues (69). The values obtained ranged from 58 ng/mg DNA in rat liver to over 1µg/mg DNA in pigeon heart. These correspond to levels of poly(ADP-ribose) with a chain length of 4 or more since smaller chain lengths were not determined. It was of interest to note that nicotinamide, an inhibitor of poly(ADP-ribose) synthetase 2.4), increased levels of poly(ADP-ribose) by up to 11 fold. This may be due to increased levels of substrate (NAD) in response to nicotinamide. In addition to quantitation of poly(ADP-ribose), antibodies have been used for the demonstration of poly (ADP-ribose) in tissue sections from various rat tissues (70). The antibodies were absorbed onto the sections which were then treated with fluorescently labelled anti-IgG antibodies. In this way, poly(ADP-ribose) was demonstrated to be exclusively in the nucleus and present in all rat tissues studied except peripheral leukocytes.

A different approach to quantitating poly(ADP-ribose) has been the use of antibodies directed against PR-AMP. In addition to producing antibodies directed against PR-AMP, the authors (71) found antibodies which reacted with poly (ADP-ribose). Recently, the former have been used to estimate levels of poly(ADP-ribose) after digestion with phosphodiesterase. The values obtained were ten-fold higher than those obtained with anti-poly (ADP-ribose) antibodies (72).

Since these antibodies will react with PR-AMP derived from (ADP-ribose) $n \geq 2$ they probably represent a more realistic estimate. This is also probably an underestimate because they will not include the terminal adenine (which is converted to 5'-AMP).

Besides poly(ADP-ribose) and PR-AMP, polyA.polyU. is also effective in raising antibodies directed against poly(ADP-ribose) (73). This may be significant since a similar pattern of reactivity has been observed in 40% of the sera of patients with systemic lypus erythematosus. A similar proportion of patient's sera has similar reactivity to antibodies raised against oligo(ADP-ribose)-histone conjugates (74). Antibodies against poly(ADP-ribose) in the sera of patients with SLE were first observed by Sugimura's group (75). The finding was subsequently confirmed by Okolie & Shall (76) who suggested such antibodies may be a more reliable diagnostic test for the disease than the presently used anti-ds DNA antibody.

The quantification of mono-ADP-ribose protein conjugates in vivo has been carried out exclusively by Hilz's group. The earliest method was an isotope dilution method, similar to the one described above for poly(ADP-ribose), to determine hydroxylamine sensitive mono-ADP-ribose. The values obtained were 5.28 nmoles/mg DNA for adult rat liver, 2.19 nmoles/mg DNA for neonatal liver and 2.11 nmoles/mg DNA for Zajdela hematoma cells (77).

The second method was an optical test based on the estimation of 5'-AMP derived from the alkaline hydrolysis of ADP-ribose or ADP-ribose-protein conjugates. The value obtained for hydroxylamine sensitive mono ADP-ribose was approximately 1nmole/mg DNA in E.A.T. cells. This value was independent of the point in the growth cycle (78).

The final method, and the one currently in use, is a radioimmunological assay based on antibodies directed against 5'AMP (79,80). Values for stationary phase E.A.T. cells were 1.66 nmoles/mg DNA (total i.e. alkali labile) and 0.48 nmoles/mg DNA (hydroxylamine-sensitive). The corresponding values for rat liver were 5.3 and 1.26 n moles/mg DNA.

1.2.4 Synthesis of poly (ADP-ribose)

As stated previously the first report of ADP-ribosylation was by Chambon et al (14) who observed the incorporation of [^{3}H]-ATP into acid-insoluble material by rat liver isolated nuclei in the presence of NMN but in the absence of other nucleotides. The true substrate for the enzyme was later shown to be NAD (11-13) and since that time the synthetic enzyme, poly(ADP-ribose) synthetase, has been assayed by the incorporation of radioactive substrate into acid insoluble material. A summary of the assays used has recently been published (81). The enzyme is principally located in the nucleus. Mitochondria have been reported to possess ADP-ribose transferase activity (82). This differs from

the nuclear enzyme in that mono ADP-ribose is transferred to a single protein and this activity is unaffected by thymidine, a potent inhibitor of the nuclear enzyme (see below). Recently, Koide reported an enzyme similar to the nuclear enzyme in the mitochondria of Xenopus laevis following germinal vesicle breakdown (83). The physiological significance of this is unknown. Smulson (84) has also reported the presence of what is probably nascent poly (ADP-ribose) synthetase associated with ribosomes.

Poly(ADP-ribose) synthetase has been characterized from a wide range of eukaryotic organisms ranging from the myxomycete, Physarum polycephalum (85), to human cells (86). The majority of characterizations have been carried out using isolated nuclei. The specificity for β-NAD is high; $NADH_2$, NADP, $NADPH_2$ and α-NAD will not act as substrates. Suhadolnik et al (87) has looked at the effect of various NAD analogues with altered adenine or ribose moieties. Nicotinamide tubercidin dinucleotide, 2'(AMP) deoxy NAD and 3'(AMP) deoxy NAD were all incorporated, but the average chain length was lower than with NAD. The fact that 2'deoxy NAD is incorporated into oligomeric chains is surprising because the 2'hydroxyl (normally involved in polymer formation) is absent. It is thought the enzyme may be able to make a 1'-3" glycosidic bond. 2'deoxy and 3'deoxy NAD are interesting because they ADP-ribosylate predominantly non-histone proteins in HeLa cells and rat liver whereas NAD predominantly ADP-ribosylates histones in these nuclei (43). The mechanism by which this

occurs is not understood. Finally, Farina et al (88) have shown that the fluorescent etheno NAD is incorporated onto proteins in bull or rat testis nuclear extracts.

The synthetase requires Mg^{++} for activity and possesses an essential thiol group as evidenced by its inhibition by DTNB, inorganic and organic mercury and its stimulation by β-mercaptoethanol or DTT. The pH optimum is in the range 7.5-8.5 and the optimum temperature is 10^{o}-15^{o}C below the optimal growing temperature for the organism. The enzyme is tightly bound to DNA requiring 0.5M NaCl to extract it. The enzyme possesses an absolute requirement for DNA and is further activated by fragmented DNA. This observation is based upon the stimulation of enzyme activity in nuclei seen after limited nuclease digestion (89,90). Recently Gill showed that in a reconstituted system with supercoiled plasmid DNA, the activity of the enzyme was stimulated if one nick was introduced with Eco R1 restriction endonuclease and greatly stimulated if over 20 nicks were introduced with Hae III endonuclease (91).

The enzyme does not obey normal Michaelis-Menten kinetics. The enzyme from both BHK cells (40) and wheat embryos (92) is activated at high substrate concentrations. In contrast, the enzyme from rat pancreas is apparently inhibited by high concentrations of NAD (93). Two different Km values were obtained for the enzyme from HeLa cells in log and stationary phase (94). Dietrich & Siebert (95) observed two different Km values for hydroxylamine sensitive

and resistant ADP-ribosylation. Whether these data are indicative of two distinct enzyme activities or is simply a reflection of the same enzyme attaching ADP-ribose to a mixture of proteins by two different linkages with different efficiencies is unknown at present.

The enzyme is inhibited by a wide variety of compounds. Nicotinamide is a competitive end product inhibitor (96, 97). Various analogues such as benzamide and pyrazinamide are also good inhibitors (98). The amide group appears to be important for binding to the enzyme since nicotinic acid and 3-acetylpyridine are not inhibitors (96). Substrate analogues such as α-NAD and $NADH_2$ have also been shown to inhibit (96). Thymidine is a very good inhibitor as is its close analogue 5-bromouracil. The equivalent free bases and mononucleotides also inhibit but are less potent (96). Methylated xanthines and cytokinins have been shown to inhibit the enzyme from 3T3 cells (99). 1-methyl adenine formycin B and showdomycin inhibit the enzyme from quail oviduct (100). The most recent report is from Pearson's group who showed 2-amino-1,3,4-thiadaizole is a good inhibitor (40).

Polyamines have been shown to stimulate ADP-ribosylation in isolated nuclei from various tissues (46,100-103). A differential effect was reported in the presence and absence of Mg^{++}. Non-histone proteins were predominantly modified in the presence of Mg^{++} whereas histones were predominantly modified in the absence of Mg^{++} (101). Perella and Lea (102) showed spermine caused increased

ADP-ribosylation of H1 with concomittant decreased ADP-ribosylation of core histones. These effects cannot be explained by inhibition of degradative enzymes since Whish's group (46) showed that polyamines caused no inhibition of poly(ADP-ribose) glycohydrolase in isolated nuclei from wheat seed. Polyamines appear to cause changes in ADP-ribosylation by altering the accessibility of acceptor proteins rather than stimulating poly(ADP-ribose) synthetase.

Various groups have attempted to purify the enzyme from a variety of sources. Early attempts are summarized in Hilz & Stone (1). Since that review was published numerous other procedures have been developed and these are summarized in Table 1. The first instance of affinity chromatography being used was reported by Khan & Shall (104). A purification of 85 fold was achieved using immobilized nicotinamide as an affinity ligand and 34 fold with Blue dextran. In both cases, the apparent yield of enzyme activity was over 100%. This may be due to removal of degradative enzymes or, as the authors suggest, an endogenous inhibitor. The affinity of the enzyme for DNA was first exploited by Kristensen & Holtlund (112). They purified the enzyme 131-fold by extraction with 0.175M phosphate extraction and DNA cellulose column chromatography. An additional isoelectric focussing step was later included (113). This gave a final purification of 700-fold. A similar result was obtained if Blue dextran affinity chromatography was used in place of isoelectric focussing

Table 1 Purification of poly(ADP-ribose) synthetase from various sources

Tissue	Purification (fold)	Yield (%)	Reference
Pig thymus	85	>100	104
	34	>100	104
	2,500	50	105
	7,525	70	106
	9,235	46	107
Calf thymus	550	3-4	108
	3,000	6	109
	1,300	10-20	110
	5,000	15	57
	1,250	14	111
Ehrlich Ascites	131	33	112
	700	25	113
HeLa	454	24	115
CLL cells	no data given		58

(114). The majority of purification procedures in Table 1 have used similar techniques at some stage. Tsopanakis et al (105) and Ellison (58) observed the final enzyme preparation was extremely labile. To minimise inactivation during purification, Tsopanakis et al, later empolyed organic solvents (50% v/v ethylene glycol) at 4°C (106) and at -10°C to achieve higher specific activities (107).

The molecular weight of the enzyme has been shown to vary from 62,000 in pig thymus (107) to 130,000 in calf thymus (110). The most exhaustive characterization of the enzyme has emerged from Hayaishi's laboratory (111). The enzyme from calf thymus had a molecular weight of 110,000 and sedimentation data suggests it is a globular protein with slight assymetry. The enzyme has a pI of 9.8 and amino acid analysis showed more lysine than arginine residues. The amino acid analysis also revealed a higher content of acidic amino acid than basic. In view of the pI the majority of these are probably amides in the native protein. The amino terminal of the enzyme appears to be blocked; this is in agreement with the finding for the pig thymus enzyme (107). Recently, the enzyme from E.A.T. cells has been characterized and similar results were obtained to those for the calf thymus enzyme (114). The only major difference was for the sedimentation coefficient which gave an apparent molecular weight of over 500,000. The authors suggested the active enzyme may be an oligomer.

A variety of groups have studied the effect of DNA and histones on the activity of preparations of poly(ADP-ribose)

synthetase. DNA is known to stimulate ADP-ribosylation when added to enzyme. Yoshihara's group isolated a small fraction of DNA from a partially purified enzyme preparation by hydroxylapatite chromatography (110). They compared the ability of this fraction to stimulate the enzyme with that of commercial calf thymus DNA by enzyme saturation studies. Calculations revealed maximal activity at approximately 1 enzyme molecule to 200 base pairs with calf thymus DNA, 40 base pairs with poly dA:poly dT and only 10 base pairs with the DNA isolated during the purification procedure. This "active" DNA has been partially characterized (110,116). It has a molecular weight of 200,000 and a G:C content of 43%. A similar fraction has been isolated by Mandel's group using a different purification procedure (117). Yoshihara's group was able to show that in the presence of DNA, but absence of any exogenous acceptor, the enzyme ADP-ribosylated itself (55). They were able to demonstrate a change in the mobility of the enzyme on SDS gel electrophoresis with varying times of incubation. By performing pulse chase experiments, they, together with Nolan (118), showed that elongation of poly(ADP-ribose) occurred by the addition of successive ADP-ribose units to the AMP terminus of the growing chain.

Histones are known to activate poly(ADP-ribose) synthetase. Yoshihara et al (110) showed that although histones stimulated enzyme activity 100% or 400% with intact or partially denatured calf thymus DNA respectively, the enzyme was nearly maximal even without histones if poly dA:

poly dT or "active" DNA were used in the assay; stimulation by histones was less than 25%. Hayaishi's group also found histones stimulated poly(ADP-ribose) synthetase activity but when the reaction product was analysed by gel electrophoresis they could not detect ADP-ribosylation of histones (57). It was suggested that the activation by histones may be due to their masking inhibitory sections, possibly single stranded, of DNA. They later showed that the purified enzyme preparation could incorporate ADP-ribose onto a mono(ADP-ribose)-H1 conjugate (119). This suggested that either a separate enzyme was responsible for initiation or the activity was lost during the purification. Recently, however, the same group reported that the enzyme could both initiate and elongate oligo(ADP-ribose)-histone H1 conjugates (120) by using higher H1 and DNA levels. In this report, they also confirmed Yoshihara's observation that the enzyme itself could act as an acceptor. Mandel et al have made a comparison of the purified enzyme preparation prior to removal of active DNA with a reconstituted preparation. The DNA-independent enzyme (prior to hydroxylapatite chromatography) was able to ADP-ribosylate all the histones, the enzyme itself, purified cholera toxin, E.coli RNA polymerase and calf thymus RNA polymerase B, but not calf thymus RNA polymerase A or C (117,121). In contrast, even after the addition of "active" DNA to the reaction, the DNA-dependent enzyme (after hydroxylapatite) could ADP-ribosylate H1 but none of the other histones (121). Yoshihara's group found that a purified enzyme would only ADP-ribosylate histone H1 if Mg^{2+} was omitted from the reaction mixture (122).

As can be seen from the above data. It is difficult to interpret the results obtained from a reconstructed enzyme system. Indeed the validity of poly(ADP-ribose) synthetase activity measurements in isolated nuclei has recently been questioned. Shall argues that because the enzyme is activated by damaged DNA and during the isolation of nuclei the DNA is damaged, the results obtained from isolated nuclei represent an overestimate of the activity of the enzyme at the time the cell was harvested (123). For this reason, a nucleotide permeable cell system was used for enzyme activity measurements. By using such a system, Shall's group showed that the activity in the permeable cell was much lower than in an equivalent number of isolated nuclei. If they were pre-incubated in the absence of NAD, the activity increased approaching those values found in isolated nuclei. This increase in activity could be prevented if the cells were pre-incubated in the presence of factors required for DNA repair. Berger's group, using a different permeabilization technique, had previously shown that incorporation of dTTP into permeabilized mouse L-cells represented semi-conservative replication, whereas in isolated nuclei this was not the case (124). They performed a full characterization of ADP-ribosylation carried out in permeable L-cells (125). In agreement with Shall, Berger observed a low rate of ADP-ribosylation in permeabilized cells. This was sensitive to known inhibitors of poly(ADP-ribose) synthetase. They could increase the rate dramatically by pre-incubation with DNaseI

and Triton X100. This later they called 'total' activity and non-pretreated activity 'intrinsic' activity. Results obtained in other systems using this system will be presented in the relevant sections below.

1.2.5 Degradation of poly(ADP-ribose)

Poly(ADP-ribose) has two bonds which are known to be susceptible to enzymic hydrolysis. The pyrophosphate bond is cleaved endonucleolytically by a phosphodiesterase from Crotalus adamenteus producing phosphoribosyl-AMP and 5'AMP (11,13,20). Exonucleolytic cleavage from the AMP terminus is mediated by a phosphodiesterase from rat liver (126-128). A phosphodiesterase, of unknown specificity, from cultured Nicotinia tabacum (129,130) also cleaves the polymer. However, DNase, RNase, micrococal nuclease, spleen phosphodiesterase and nucleotide pyrophosphatase have no effect on poly(ADP-ribose) (131).

The ribose-ribose bond is cleaved by an enzyme called poly(ADP-ribose) glycohydrolase. This enzyme was first discovered in the nuclei of calf thymus by Miwa and Sugimura (132) who found it to be mainly associated with chromatin. It has been purified 200-fold with 5% yield and cleaves poly(ADP-ribose) exonucleolytically leaving the terminal ADP-ribose attached to protein. The enzyme was inhibited by cyclic AMP, ADP-ribose and p-chloromercuribenzoate, (133). Ueda *et al* (134) have shown poly(ADP-ribose) glycohydrolase exists in a rat liver nuclei soluble

fraction. This enzyme appears to be identical with enzyme found in the insoluble chromatin fraction (135). The enzyme has also been shown to exist in mouse L-cell nuclei (136), Physarum polycephalum (137), in wheat embryo (138) and in rat testis (139). A comparison of properties is found in Tanaka et al (137). There appear to be two enzymes present in rat testis as based on different pH optima and KCl stimulation. Miwa et al (140) have shown that in various rat tissues poly(ADP-ribose) glycohydrolase is the major degradative enzyme for poly(ADP-ribose).

DNA has been shown to inhibit poly(ADP-ribose) glycohydrolase in several systems see ref. (1) . Denatured DNA is an extremely potent inhibitor of the enzyme. Double stranded DNA has a variable inhibitory effect which may simply be a reflection of the single stranded DNA impurities in the preparations used. Stone et al (141) showed that the enzyme was bound more tightly to denatured DNA-cellulose than to double stranded DNA-cellulose. They also showed that inhibition of the enzyme by high levels of DNA could be overcome by either increasing the ionic strength of the medium or by adding histone H1 and they suggested that both of these treatments may be displacing the enzyme from inhibitory (possibly single stranded) portions of DNA.

Synthesis of H1 'dimer' (49) in nuclei of various cell types showed an inverse correlation with poly(ADP-ribose) glycohydrolase activity (52). Thus, HeLa cell nuclei, producing H1 'dimer', possessed no detectable poly(ADP-ribose)

glycohydrolase activity whereas MTW-9 rat mammary nuclei had high levels of enzyme but no dimer. It was shown that calf thymus poly(ADP-ribose) glycohydrolase purified 200-fold by the method of Miwa et al,(101) hydrolysed H1 dimer from HeLa at one ninth the rate of an equivalent mass of poly(ADP-ribose)$_{15}$. The rate of degradation of the 'dimer' was increased two-fold by the addition of DNA at a ratio of 2:1 with H1, suggesting DNA may cause a conformational change in the structure of the 'dimer'.

Various authors have observed that poly(ADP-ribose) glycohydrolase does not remove the final ADP-ribose residues from protein (106, 101, 49). Recently, Okayama et al, (142) have shown the presence of an enzyme from rat liver nuclei which could remove ADP-ribose from mono (ADP-ribose)-H2B conjugates. The released product was similar but not identical to, ADP-ribose. The enzyme has been partially purified from rat liver. It has a pI of 4.9 and molecular weight of 240,000. It is inhibited by ADP-ribose and DNA and is able to cleave mono but not poly(ADP-ribose) from various proteins (143).

1.3 Biological role of ADP-ribosylation

Since Hogeboom & Schneider(144) first discovered that NAD pyrophosphorylase was located exclusively in the nucleus; various authors have suggested that NAD fulfills a role in addition to that of respiratory coenzyme. As early as

1958, Morton (145) suggested it may hold a key role in the regulation of cell proliferation in that the nucleus may sense changes in intracellular NAD levels. The discovery in 1966-7 that a nuclear enzyme, poly(ADP-ribose) synthetase, utilised NAD as substrate for the modification of nuclear proteins suggested that such an enzyme may provide the mechanism by which NAD levels are sensed. The precise biological role of poly(ADP-ribose) synthetase remains unclear despite a large amount of research. That this enzyme plays an important physiological function is suggested by the high turnover of NAD in eukaryotic cells. In human D98/AH2 cells, Reichsteiner et al (146) have shown that the half life of NAD was about 1 hour. This means that of the NAD synthesized in the cell only 5% is required for maintenance of the NAD concentration in the cell; the remaining 95% is hydrolysed. They had previously shown that, in cells enucleated by cytochalasin, the half life of NAD was 10 hours (147). From these data, it was concluded that the nucleus was the major site of degradation of NAD and suggested that poly(ADP-ribose) synthetase was chiefly responsible. It has since been shown that in E.coli, which does not contain poly(ADP-ribose) synthetase, NAD is degraded primarily at the pyrophosphate linkage, whereas in D98/AH2 cells the majority of NAD is cleaved at the N-glycosidic linkage (148). This finding is consistent with the idea that poly(ADP-ribose) synthetase is the major degradative enzyme for NAD.

The approaches used to elucidate the physiological role of ADP-ribosylation have been varied. The earliest method was to attempt to correlate the activity of poly (ADP-ribose) synthetase in isolated nuclei with cells under various conditions. As stated previously, the validity of using isolated nuclei to perform enzyme assays is uncertain. Studies using permeabilized cells are therefore probably more realistic.

The treatment of cells with inhibitors of poly(ADP-ribose) synthetase with a view to examining them for cellular dysfunction also leads to ambiguous results because no inhibitor used to date is physiologically specific for poly(ADP-ribose) synthetase (for a detailed discussion, see section 4). The most direct approach has been the correlation of levels of protein ADP-ribosylation in vivo with various cell types.

A major problem in ascribing a precise role for ADP-ribosylation is the difficulty in correlating the data obtained by the above methods with a defined cell function e.g. the increase in poly(ADP-ribose) at the S/G_2 boundary of HeLa cells may reflect inhibition of DNA synthesis or it may be involved in subsequent chromatin condensation.

For these and other reasons, attempts to define the biological role of ADP-ribosylation have produced wide ranging and contradictory suggestions for its function. The data obtained is presented below under the function the authors have suggested it may fulfill.

1.3.1 DNA synthesis and cellular proliferation

In 1970, Burzio & Koide (149) showed that pre-incubation of rat liver isolated nuclei with NAD caused a decrease in the incorporation of ^{3}H -TTP into acid-insoluble material. This decrease was prevented if the nuclei were pre-incubated with nicotinamide and NAD. This finding was suggestive of a role for ADP-ribosylation in the control of DNA synthesis. Many workers have since examined other systems and have found conflicting results. ADP-ribosylation either decreases, has no effect on or increased dTTP incorporation. The various results are summarized in Hilz & Stone (1).

Since that time Janakidevi (150) has shown that removal of lysine-rich histones or treatment with heparin increases DNA synthesis in swine aorta isolated nuclei. The decrease in poly(ADP-ribose) synthesis observed with lysine-rich histone is explained by co-extration of poly(ADP-ribose)-synthetase and they conclude that removal of lysine-rich histones or poly(ADP-ribose)-synthetase exposes initiation sites for DNA synthesis. Tanigawa's group have shown that pre-incubation of isolated nuclei from chick embryo liver with NAD increased dTTP incorporation into NAD. Conversely, NAD pre-incubation of nuclei from hen liver decreased dTTP incorporation. Extraction of NAD-treated embryo and adult liver nuclei with 0.35M NaCl with subsequent reconstitution showed that the factors responsible for suppression or stimulation of DNA synthesis were present in the 0.35M NaCl extract (151). They later

showed the stimulation of DNA synthesis observed with chick embryo nuclei was due to increased accessibility of the DNA to nuclease (152). Administration of glucocorticoid hormone to chick embryos caused decreases in both DNA and poly (ADP-ribose) synthesis (153). This may be due to loss of enzyme activity as a result of increased nuclear fragility.

Miwa et al (154) showed that poly (ADP-ribose) synthetase activity was 2 to 10 fold higher in nuclei from SV40 transformed cells versus untransformed cells. They also demonstrated that in contrast to untransformed cells, where enzyme activity remained constant, the activity in transformed cells increased markedly throughout the growth cycle. Muller et al (155) found that alteration of DNA synthesis by Herpes Simplex Virus (HSV) infection was not accompanied by a change in poly(ADP-ribose)-synthetase activity in BHK cells suggesting ADP-ribosylation plays no role in the control mechanisms for cellular or HSV DNA synthesis.

Ghani and Hollenberg (156) demonstrated that chick embryo heart cells exhibited higher poly(ADP-ribose) synthetase activity in isolated nuclei from cells grown in 20% (v/v) O_2 than from cells grown in 5% O_2. They later showed, by pulse labelling the cells with ^3H adenosine, that poly(ADP-ribose) levels in vivo were higher in cells grown in 20% (v/v) O_2 (157). They suggested that in rapidly growing heart cells (5% O_2), the redox potential shifts i.e. $NAD^+ \rightarrow NADH_2$, causing decreased ADP-ribosylation and thus an increase in DNA synthesis.

Suhadolnik's group looked at the ability of exogenously added E.coli DNA polymerase 1 to incorporate ^{3}H -dTTP into nuclei from rat liver or HeLa cell nuclei which had been pre-incubated with either NAD or 2' or 3' deoxy NAD (87). 0.5mM NAD caused only a 9% decrease in TTP incorporation into adult rat liver isolated nuclei, whereas 2' (or 3') deoxy NAD inhibited incorporation 90%. A similar result was found in both foetal rat liver and Novikoff hepatoma nuclei although the inhibition by deoxy NAD was less (50%). In HeLa cells, NAD caused a 2.5 fold stimulation of incorporation but deoxy NAD resulted in a slight inhibition. They later showed that this was due to a difference in the acceptor proteins for NAD and deoxy NAD (43). Incubation of both rat liver nuclei and HeLa cell nuclei resulted predonimantly in modification of histones whereas deoxy NAD mainly modified non-histone proteins. The physiological significance of these data is not understood.

For the reasons detailed in section 2.4, isolated nuclei are clearly far from ideal as <u>in vivo</u> models for the study of ADP-ribosylation. In addition it must be questioned whether incorporation of dTTP into isolated nuclei represents semi-conservative DNA synthesis. Berger's group have shown that incorporation of dTTP into permeabilized L-cells does represent semi-conservative DNA synthesis (124) and have since looked at the effect of ADP-ribosylation in these cells on DNA synthesis. They showed that ADP-ribosylation had no effect on DNA synthesis and vice versa when both processes occurred simultaneously. An increase in the

rate of ADP-ribosylation was seen as the cells progressed through the growth cycle; this was in agreement with the data of Stone & Shall (158) obtained in isolated nuclei. If, however, the activity was measured in the presence of DNase 1 and Triton X100, no change in the rate of ADP-ribosylation occurred. The significance of this will be discussed in section 1.3.4. In the following paper it was shown that after DNA synthesis was inhibited by acute glucose deficiency, vaccinia virus infection or cytosine arabinoside treatment, the intrinsic activity of poly(ADP-ribose) synthetase increased (159).

Rates of ADP-ribosylation and DNA synthesis in permeabilized cells were studied following phytohaemagglutinin (PHA) treatment of normal and chromic lymphocytic leukaemic (CLL) lymphocytes (160). Lehmann et al (161) had previously shown that ADP-ribosylation in isolated nuclei increased three-fold following treatment of pig lymphocytes with PHA. Berger et al, using the permeabilized cell assay found that both normal and CLL lymphocytes showed an increase in ADP-ribosylation upon treatment with PHA. This effect appears to be divorced from DNA synthesis after PHA stimulation of CLL lymphocytes, the response of DNA synthesis was both much lower and occurred at a later time than in normal cells (160). The methodology employed by Berger for determining the rate of ADP-ribosylation has recently been questioned by Wielckins et al (162). Berger had found both intrinsic activity and total activity were increased in CLL lymphocytes when compared to normal lymphocytes; an increased activity had previously

been observed in CLL lymphocytes by Koide's group when assayed in isolated nuclei (163). Wielckins et al observed that measuring initial rates rather than incorporation of [^{3}H]-NAD after a fixed period of time (30 min) resulted in no difference between intrinsic activity in normal and CLL lymphocytes but a 2.5 fold stimulation in total activity when measured by either permeabilized cells in the presence of DNase or in homogenates. These results, and the difference in kinetics of ADP-ribosylation observed both by Wielckins et al and Koide's group, may be explained by the lower levels of mono-ADP-ribose conjugates in CLL lymphocytes in vivo (162). Thus in normal lymphocytes, saturation of protein acceptors may limit both the rate and the extent of ADP-ribosylation observed in permeabilized cells, and in isolated nuclei. Evidence to support this view has been obtained by Yamada's group who showed that after synchronization of cells with thymidine, a known inhibitor of poly(ADP- ribose) synthetase in vitro, much higher levels of ADP-ribosylation were observed in isolated nuclei immediately following release of the thymidine block than in cells synchronized with either hydroxyurea or amethoptein which do not inhibit poly(ADP-ribose) synthetase (164). They concluded that thymidine (and nicotinamide which gave a similar result) was inhibiting ADP-ribosylation in vivo and thus providing more acceptor sites for the in vitro assay.

An interesting correlation was observed when Bredehorst et al (165) studied ADP-ribosylation in permeabilized cells during the growth cycle of E.A.T. cells. In agreement with

Berger's data for L-cells, they observed an increase in intrinsic, but not total, activity as the cells passed from a proliferative to non-proliferative stage. They observed an increased level of total mono(ADP-ribose)-protein conjugates in non-proliferating cells in vivo. This increase in total levels was a result of increased levels of hydroxylamine-resistant residues; hydroxylamine sensitive residues remained relatively constant. This is the first data to suggest ADP-ribosylation may fulfill two different functions.

3.3 DNA transcription

As in the case of DNA replication, two main approaches have been used to investigate a possible role for ADP-ribosylation in the transcription of DNA viz the effect of pre-incubation with NAD on the ability to incorporate radioactive precursor into acid-insoluble material and secondly the correlation of poly(ADP-ribose) synthetase activities with the ability of various cells to synthesize RNA.

The first approach was used by Burzio & Koide who observed no decrease in ^{3}H-UTP incorporation into rat liver nuclei after pre-incubation with NAD (166). Furneaux & Pearson (167) investigating the effect of ADP-ribosylation on the ability of BHK cell nuclei found two opposite results depending on the ionic strength of the reaction mixture. At low ionic strength, ADP-ribosylation diminishes the ability

of nuclei to incorporate ^{3}H -UTP whereas at high ionic strength UTP incorporation is stimulated. They suggest that the differential effects are due to the two forms of RNA polymerase; RNA polymerase I which is more active at low ionic strength and RNA polymerase II which is more active at high ionic strength. Investigation of the inhibition at low ionic strength showed that the α-amanitin resistant polymerase (I) was more sensitive to ADP-ribosylation than the total polymerase activity. They further suggest that the inhibition was due to ADP-ribosylation of RNA polymerase 1 because when assayed using an exogenous DNA template, the polymerase extracted from NAD treated nuclei had lower activity than that from control nuclei. Direct ADP-ribosylation of RNA polymerase 1 had earlier been suggested by Muller's group (168) who had shown that following pre-incubation with ^{3}H-NAD, RNA polymerase 1 had ^{3}H -poly (ADP- ribose) associated with it after a purification of 550-fold from nuclei. The amount of poly(ADP-ribose) associated with RNA polymerase I showed an inverse correlation with the transcriptional activity of the oviduct from which it was isolated.

The second approach for establishing a definite role for ADP-ribosylation in DNA transcription, i.e. the correlation of poly(ADP-ribose) synthetase with cells of different capacities for transcription, has been also used by Muller's group. They found that during hormone induced gene expression, nuclei from immature quail oviducts had lower poly(ADP-ribose) synthetase activity than those from controls (169). This is

once more indicative of a regulatory role for ADP-ribosylation. A different result, however, was obtained using a similar approach in rat liver. Hilz & Kittler (170) could detect no difference in poly(ADP-ribose) synthetase activity in nuclei isolated from control, adrenalectromized or cortisol-treated rats.

Smulson's group have suggested that poly(ADP-ribose) synthetase is preferentially localized in transcriptionally active chromatin (171). This finding would also suggest ADP-ribosylation was involved in transcription. Yukioka et al (172) have since questioned the methodology used to separate transcriptionally active and inactive chromatin. Using a different technique they could not detect a difference in the specific activities of the enzyme between the two forms of chromatin.

The specific activity of poly(ADP-ribose) metabolizing enzymes in nucleoli, the site of ribosomal RNA transcription, has been compared with that in whole nuclei form Tetrahymena pyriformis (173). The authors could detect no difference in the specific activity of either poly(ADP-ribose) synthetase or glycohydrolase in nucleoli. A different result was obtained by Kawashima & Izawa (174) who used a autoradiographic technique to examine the site of ADP-ribosylation in nuclei from E.A.T. cells. The nucleolus appeared to have higher activity than the rest of the nucleus. It was also shown that following SDS gel electrophoresis, a different set of proteins were modified.

Among these it was found that a nucleolus-specific phosphoprotein with a molecular weight of 120,000 was ADP-ribosylated. A role for ADP-ribosylation in the regulation of ribosomal gene transcription is suggested by the finding that RNA polymerase 1 is inhibited by ADP-ribosylation (see above) and also from the observation that the protein A24, which is thought to act as a controller of ribosomal gene expression (175), is ADP-ribosylated in rat liver nuclei (45).

1.3.4. DNA repair

The study of a possible role for ADP-ribosylation in the process of DNA repair has been prompted by two observations. Firstly, it has long been known that treatments which damage DNA cause a depletion of intracellular NAD and secondly that fragmentation of DNA by nucleases stimulates the activity of poly(ADP-r bose) synthetase *in vitro* (section 1.3.4).

The first reports that treatment of cells *in vivo* with alkylating agents increased poly(ADP-ribose) synthetase activity came from Smulson's and Shall's labs. Both groups demonstrated that following treatment of cells with streptozotocin, the 2-deoxyglucose derivative of N-methyl-N-nitrosourea (MNU), the activity of poly(ADP-ribose) synthetase in isolated nuclei from HeLa cells (176) and Physarum polycephalum (177) was increased. A wide variety of alkylating agents have been shown to stimulate poly(ADP-

ribose) synthetase activity. These include MNU itself (178), N-methyl-N-nitro-N-nitrosoguanidine (63), alkyl sulphonates and alkyl sulphates (179). Smulson has shown that, in isolated nuclei, the increased ADP-ribosylation in response to MNU treatment of HeLa cells is a result of increased availability of protein acceptors rather than an increase in the length of poly(ADP-ribose) chains (180). Jacobson & Jacobson (181) examined the effect on NAD levels of N-nitroso compounds which were either direct-acting or indirect-acting carcinogens or non-carcinogens in both 3T3 cells or nitrogen-stimulated human lymphocytes. Direct-acting carcinogens were observed to cause large decreases of NAD in both cell types whereas indirect-acting carcinogens decreased levels in lymphocytes but not 3T3 cells. Non-carcinogens had no effect on NAD in either cell type. Smulson's group have compared two nitrosoureas in their ability to stimulate poly(ADP-ribose) synthetase and depress NAD levels (180). These were MNU which alkylates DNA and causes strand breaks and 1-(2 chloro-ethyl)-3 cyclohexyl-1-nitrosourea (CCNU) which carbamoylates DNA causing extensive cross linking but less strand breakage. MNU and other alkylnitrosoureas cause a decrease in cellular NAD and stimulated ADP-ribosylation *in vitro*. CCNU and other chloroethyl nitrosoureas had no effect on NAD levels and slightly inhibited ADP-ribosylation. In order to investigate this phenomenon further, the sites of action of MNU and CCNU were examined. MNU principally alkylated the internucleosomal regions of DNA, which has

been reported to be the site of poly(ADP-ribose) synthetase, whereas CCNU mainly modified the nucleosomal core DNA (192). In additon, the histones were predominantly modified by MNU and non-histones were the major site with DDNU. They suggested that the differential sites of action of these two compounds may explain the separate effects on ADP-ribosylation in isolated nuclei.

Among other treatments in vivo which are known to damage DNA, γ-irradiation (183), uv irradiation (184) and antibiotics such as neocarzinostatin (183), bleomycin (185), macromomycin and adriamycin (186) are all known to cause increase in poly(ADP-ribose) synthetase activity in vitro. Berger's group have compared the response of lymphocytes from patients with xeroderma pigmentosum (XP) to damage by either MNNG or uv-irradiation with that of normal lymphocytes (187). Cells from patients with XP are reported to be unable to repair DNA damage caused by uv-irradiation (188,189) but are able to repair damage caused by MNNG (190). Both normal and XP cells showed large increases in intrinsic poly(ADP-ribose) synthetase activity in permeabilized cells after MNNG treatment. The response was much greater in XP cells (150-300% in normal cells vs 500-1500% in XP cells). After uv irradiation, XP cells had either absent, much lower or much delayed responses than normal cells. Berger also suggested that the increased intrinsic activity seen in stationary vs. log phase cells (and in temperature sensitive cells grown at 33^{o}C) may be a result of damaged DNA rather than inhibition of DNA replication (191).

Hilz's group have demonstrated that as E.A.T. cells enter stationary phase the hydroxylamine-resistant, but not the sensitive, mono(ADP-ribose) residues increase *in vivo*. It is possible that DNA damage could be associated hydroxylamine-resistant residues and the sensitive residues with other functions. Jacobson's group (65) have recently shown that, in SV40 transformed 3T3 cells, the poly(ADP-ribose) content of these cells increased 150 fold in response to MNNG treatment.

All the data described above indicate that poly(ADP-ribose) synthetase and ADP-ribosylation are associated with the damage of DNA but none of the data proves that ADP-ribosylation is involved in its repair. Results presented below attempt to prove that ADP-ribosylation is indeed involved with DNA repair.

Shall's group have shown that the cytotoxicity of DNA-damaging treatments such as alkylating agents, γ-irradiation and neocarzinostatin (179,192) was increased when administered to cells in the presence of known inhibitors of poly(ADP-ribose) synthetase at doses which are not cytotoxic in themselves. The inhibitors used were 5-methyl nicotinamide, thymidine (which when administered with deoxy cytidine does not inhibit DNA synthesis) and methylated xanthines. It was argued that such an effect is mediated by inhibition of ADP-ribosylation *in vivo* with a consequent inability to repair the damaged DNA. They have since shown, however, that caffeine, a known

inhibitor of both poly(ADP-ribose) synthetase and post-replication repair, probably does not exert its effect via ADP-ribosylation but by some other mechanism (193).

Jacobson & Narasimhan (194) have shown that 3T3 cells depleted of NAD by culture in nicotinamide-free medium are unable to undergo carcinogen-induced unscheduled DNA synthesis (i.e. gap filling) indicating that NAD, the substrate for ADP-ribosylation, is essential for the repair of DNA. Durkez et al (195) have reached a similar conclusion after showing that NAD-depleted LS1210 cells are unable to convert low molecular weight DNA to high molecular weight DNA following treatment with dimethylsulphate (DMS). The possibility that NAD, rather than ADP-ribosylation, is involved is excluded because using 3-aminobenzamide, one of the inhibitors of poly(ADP-ribose) synthetase discovered in this laboratory, the repair of DMS induced damage was inhibited although NAD levels did not change.

The above data strongly suggests that one of the biological roles of ADP-ribosylation is the repair of strand breakes in DNA.

1.3.5 Cell cycle

Many workers have investigated the involvement of ADP-ribosylation in the timing of events during the cell cycle. The majority of studies have involved measurements of rates of ADP-ribosylation in nuclei isolated from cells in different stages of the cell cycle. A large number of

different results have emerged and most are summarized in Hilz & Stone's review (1). In general it was found that enzyme activity was low in S-phase and high in G_2 phase. Kidwell used adriamycin, macromomycin, neocarzinostatin and bischloroethyl-nitrosourea to arrest synchronized HeLa and L929 cells in G_2 phase (186). The first three of these compounds all cause single strand breaks in DNA (196,197), which have been shown to activate poly(ADP-ribose) synthetase (see previous section). Only adriamycin and macromomycin, however, caused an increased enzyme activity in isolated nuclei; neocarzinostatin, and bischloro-nitrosurea, had no effect. Furthermore the two effective compounds stimulated the incorporation of ^3H -adenosine into poly(ADP-ribose) *in vivo*. It was suggested that the different compounds were acting at different points in G_2 phase and that increased ADP-ribosylation occurred at a specific point in G_2.

Tanuma *et al* (198) used colecmid to inhibit mitosis in HeLa cells. They found that this treatment caused a five-fold increase in ADP-ribosylation in isolated nuclei. Berger's group have studied ADP-ribosylation in permeabilized chinese hamster ovary cells following mitotic selection (199). The intrinsic activity increased during G_1, decreased rapidly as the cells traversed S-phase, and then increased during G_2 , M and G_1 phases. The total activity, however, remained constant through G_1, increased during S-phase and decreased after mitosis. They suggested that the enzyme was synthesized during S-phase, as are the histones (200).

This had previously been indicated by Smulson's group who found that poly(ADP-ribose) synthetase activity was associated with cytoplasmic ribosomes during S-phase but remained low at other stages (84). This may account for the increase in activity observed by Roberts et al (201) and Colyer et al (202) during the S-phase when they used isolated nuclei for their assays.

Levels of poly(ADP-ribose) and mono(ADP-ribose) protein conjugates have been measured in vivo during the cell cycle. Kidwell & Mage (68) used a radioimmunoassay to measure a poly(ADP-ribose) throughout the cell cycle of HeLa. Levels increased six fold as the cells passed from early S to late S phase. A larger increase was seen during early G_2 phase (ten-fold). Hilz's group (203) used a radioimmuno-assay to measure mono ADP-ribose protein conjugates through the cell cycle of the naturally sunchronous slime mould Physarum polycephalum. Total conjugates decreased following mitosis through S-phase (G_1 is absent) increased sharply at the S-G_2 boundary and continued to rise slowly throughout G_2 to mitosis. When hydroxylamine sensitive and resistant residues were analysed, two distinct patterns emerged. Hydroxylamine sensitive ADP-ribose conjugates decreased immediately following mitosis, remained low through S-phase increased at the S-G_2 boundary and remained at that level except for a transient sharp decrease in late G_2. Hydroxylamine resistant residues, however, also decreased following mitosis but increased during S-phase; they then remained constant except for a transient increase during late G_2.

These data strongly suggest that ADP-ribosylation is involved in more than one function during the cell cycle of Physarum Polycephalum.

1.3.6 Cellular differentiation

Although the data linking ADP-ribosylation with differentiation is presented below, its involvement may be mediated through an effect on any of the processes mentioned previously. The two approaches used have been to study ADP-ribosylation either during the early development of an organism or during experimentally induced differentiation.

Caplan examined the differentiation of mesodermal cells from embryonic chick limb buds into either muscle or cartilage cells. He found that NAD levels fluctuated during differentiation and later examined the possible involvement of ADP-ribosylation in the process (204). Chondriogenic expression was inhibited by exposure of cultures to either nicotinamide or 5-bromo deoxy uridine, both of which inhibit ADP-ribosylation in vitro (section 1.2.4), and this treatment also caused a decrease in the incorporation of ^{3}H -adenine into poly(ADP-ribose) in vivo. Treatment of cultures with 3-acetylpyridine caused a potentiation of chondriogenic expression and a stimulation of ^{3}H -adenine incorporation into poly(ADP-ribose). The effects of 3-acetylpyridine, however, have been ascribed to the complete destruction of peripheral nerves over 24

hours exposure, resulting in a much greater repression of myogenosis than chondriogenesis although both processes are inhibited (205). Caplan, together with Mandel's group, has shown using a fluorimetric assay for poly(ADP-ribose) that the levels do fluctuate during the development of limb buds and that cartilage and muscle cells have two distinct profiles. (206).

Hilz's group have looked at mono ADP-ribose during the development of rat liver and have found hydroxylamine sensitive and resistant residues showed two separate profiles and resistant residues showed two separate profiles (unpublished data). Koide's group found that during Xenopus laevis oocyte maturation the activity of poly(ADP-ribose) synthetase rose three-fold just prior to oocyte nuclear breakdown, which is mediated by progesterone (207). They have recently reported that nuclear breakdown is associated with a decrease in nuclear poly(ADP-ribose) synthetase and an increase of a mitochondrial form of the enzyme (83). This activity appears to be distinct from the mitochondrial form reported by Kun et al (82) in rat liver. Farzenah & Pearson (208,209) have shown the ADP-ribosylation in isolated nuclei increases during the embryonic development fo Xenopus laevis and these two observations are consistent with a role for ADP-ribosylation in the early development of this organism.

Young & Sweeney (210) looked at the incorporation of ^{3}H -adenosine into RNA and ADP-ribosylated proteins in vivo during the maturation of mouse ova. They found that 7-8 hours following ovulation the incorporation of adenosine was

low which was correlated with a decrease in the fertilizability. The same authors have also investigated incorporation into mouse one-cell embryos and found that poly(ADP-ribose) in embryos had both a different chain length than that found in ova and a different sensitivity towards hydroxylamine (211). These data also point to an involvement of ADP-ribosylation in differentiation. Hayaishi's group have also suggested ADP-ribosylation may be involved in the maturation or transformation of peripheral granulocytes following immunohistochemical studies (70).

Two groups have studied the effect of induction of Friend cell differentiation on the activity of poly(ADP-ribose) synthetase activity in isolated nuclei. Rastl & Swetly (212) found that using cell line F4N, which is sensitive to butyrate-induced differentiation but relatively insensitive to hexamethylene bis acetamide (HMBA) induction, found that both compounds caused an increase in poly(ADP-ribose) synthetase activity. A similar increase was noted if the cells were allowed to accumulate in the G_1 phase of the cell cycle by either growth to high density or isoleucine deprivation. It has recently shown that his increased ADP-ribosylation is a consequence of histone, rather than non-histone protein, modification (213). Morioka et al (214), using cell line 745, found that addition of butyrate, dimethylsulphoxides or HMBA all or which induced erythropoetic differentiation, caused a decrease in poly(ADP-ribose) synthetase activity, although a slight transient increase with butyrate was observed. They also found that nicotinamide,

an inhibitor of poly(ADP-ribose) synthetase activity *in vitro*, induced differentiation in these cells as had Rastl & Swetly (212). When co-administered with the other compounds, it was found to inhibit butyrate induction but enhanced induction by DMSO and HMBA. The significance of this result will be discussed later. Terada *et al* (215) have demonstrated that a variety of compounds which are known to inhibit poly(ADP-ribose) synthetase *in vitro* also induce differentiation of Friend cells. The finding that N-methyl-nicotinamide was the most potent inducer tested suggest that inhibition of poly(ADP-ribose) synthetase is not essential. With this exception however, the ability to induce differentiation showed a rough correlation with the inhibitory potency of the compounds tested.

Finally it was shown that addition of poly(ADP-ribose) itself to mouse myeloid leukaemic cells induced differentiation although the significance of this finding is uncertain because dextran sulphate or poly vinyl sulphate caused a similar effect and it is also questionable whether poly(ADP-ribose) would cross the membrane in an intact form (216).

1.3.7 Other possible functions of ADP-ribosylation

In addition to the above stated postulated roles, three other possible functions have been suggested.

1. The obervation that nicotinamde, 5-methyl nicotinamide, thymidine and 3-isobutyl-1-methylxanthine induce ornithine decarboxylase (EC 4.1.1.17) has prompted Minaga *et al* (217)

to suggest ADP-ribosylation may be involved in the regulation of this enzyme. This is an intriguing observation because the product of ornithine decarboxylase, spermine, has been shown to stimulate poly(ADP-ribose) synthetase in isolated nuclei (see section 1.2.4). Recently Bredehorst et al (165) have shown an inverse correlation between ornithine decarboxylase and poly(ADP-ribose) synthetase activity as measured by the permeabilized cell system during the growth cycle of E.A.T. cells.

2. Suzuki & Murachi (218) have shown that a nucleic acid-like inhibitor co-extracts with a chromatin-bound neutral protease from rat peritoneal macrophages. They found that inhibition of protease activity could be removed by pre-incubating the de-proteinized inhibitor fraction with a crude poly(ADP-ribose) glycohydrolase preparation from rat liver before addition to the assay mixture. DNase 1, P1 nuclease and snake vanom phosphodiestase had no effect. From this they suggest that the inhibitor is similar but not identical to poly (ADP-ribose). It was also shown that poly(ADP-ribose) with an average chain length of 30 residues was inhibitory.

3. Matinyan & Umanskii (219-220) have shown that the 'polypeptide synthetase' activity of rat liver chromatin was enhanced by pre-incubation with NAD. This enhancement was inhibited by the presence of nicotinamide or thymidine. On storage of chromatin for 20 hours at $4^{o}C$, a complete loss of polypeptide synthetase activity was observed.

This, however, could be prevented by the presence of cyclic 3'-5' AMP, an inhibitor of poly(ADP-ribose) glycohydrolase. They suggest that poly(ADP-ribose) may serve as an energy source for amino-acid activation.

1.3.8 Possible mechanisms by which ADP-ribosylation may affect cellular processes.

Listed below are some of the most obvious mechanisms by which ADP-ribosylation may affect cellular processes.

1. Modification of an enzyme resulting in inhibition as proposed by Muller & Zahn (168) and Furneaux & Pearson (167) for RNA polymerase 1 and by Koide's group (54) for a Ca^{2+} Mg^{2+} dependent endonuclease. This could occur either by ADP-ribosylation at the active site or by altering the affinity for DNA.

2. Modification of a enzyme resulting in a change of specificity such as occurs when E.coli RNA polymerase 1 is ADP-ribosylated during T4 infection (1).

3. Modification of a regulatory protein such as A24 (45).

4. To act as a signal in response to a change in the environment of poly(ADP-ribose) synthetase. This has been postulated to occur during DNA damage and may act either directly or by exposure to damaged DNA to repair enzymes. This is supported by Smulson's

observation that poly(ADP-ribose) synthetase activity is principally associated with extended chromatin undergoing DNA replication or repair and that ADP-ribosylation of histones renders them more susceptible to nuclease attack (221). Koide's group (222) have shown that heavily modified histone H1 has lower affinity for DNA than unmodified H1 which is also consistent with the possibility that ADP-ribosylation exposes DNA.

5. Alteration of chromatin structure. This could occur at two levels, either localized (see 4) or gross changes such as occur during chromatin condensation prior to mitosis. Smulson's group have investigated the effect of chromatin structures on the activity of poly(ADP-ribose) synthetase. It was located on the internucleosmal DNA (171, 223) and the specific activity of the enzyme increased with increasing nucleosome number up to 8-10 whereupon it decreased and then levelled off (221,224,225). They suggest that the enzyme is located on chromatin at a periodicity of 8 nucleosomes or in the middle of a 16-nucleosomes structure and this is consistent with the number of nucleosomes in the turn of the solenoid proposed by Finch & Klug (226). The major acceptor in such structures is a protein with a molecular weight of 110,000. In 8N polynucleosomes this accounts for 90% of the modification observed but this

decreases with nucleosome number in structures of lower complexity (225). This protein has been shown to co-purify with poly(ADP-ribose) synthetase and it has a similar molecular weight suggesting that the enzyme is undergoing self modification (115,227). ADP-ribosylation of histones also increases with increasing nucleosome number. In mono-, di- or trinucleosome structures, the principal acceptors are HMG proteins and proteins M1 and M4 whereas in nuclei the major acceptors are H1 and H2B (221).

In view of the role of H1 in the maintenance of higher ordered chromatin structures (226, 228-230) the discovery of the formation of a complex of 2 H1, molecules joined by poly(ADP-ribose) by Kidwell's group (49) presents intriguing possibilities in that such a structure may link 2 H1 molecules on non-adjacent linker regions of DNA. The affinity of the complex for DNA is the same as that for DNA which is consistent with a cross-linking role (141). The increased synthesis of the complex upon chromatin condensation by polyamines (231) and in poly nucleosomes having 8 or 16 nucleosomes (50) is also consistent with the above. The transient nature of this modification is inferred from the inverse correlation with poly(ADP-ribose) glycohydrolase activity (52) and the low levels of poly(ADP-ribose) in vivo (68). Until, however, the formation of the

H1 complex is demonstrated *in vivo*, the above mechanism must remain a speculation.

6. In addition to the mechanisms outlined above, at least one other possibility remains namely the protection of protein from proteolytic attack and this will be discussed in section 4.4.

1.4 Rationale for the present work

As can be seen from the previous section, a large number of contradictory results have been obtained regarding the involvement of ADP-ribosylation in the various physiological processes investigated. This could be due to a number of reasons. Firstly, there is a problem in the correlation of specific activity of poly(ADP-ribose) synthetase obtained *in vitro* with rates of ADP-ribosylation *in vivo*. The activity of the enzyme *in vitro* is dependant on a number of factors such as amount of enzyme present, extent of damage done to DNA and the number of available protein acceptors present. An increase in the activity of poly(ADP-ribose) synthetase could result from changes in any of these parameters. The permeabilized cell technique has been reported to more closely reflect the situation *in vivo* (123,125). This does not, however, rule out changes in the state of the protein acceptors. Indeed, Hilz's group have shown that an increase in intrinsic activity during the growth cycle of E.A.T. is associated with higher levels of mono ADP-ribose *in vivo* whereas in CLL cells which possess the same intrinsic activities as normal lymphocytes have

lower levels of mono ADP-ribose *in vivo*. Tanuma *et al* (164) have shown that treatment of cells with inhibitors of the enzyme gives rise to an increase in the specific activity of the enzyme in isolated nuclei. Thus, unlike other "conventional" enzymes, the assay of the enzyme is complicated by the presence of one of its substrates i.e. unmodified protien, some of its products (ADP-ribosylated protein) and a variable activator namely DNA in various states of damage.

Another approach which has been used is the measurement of total mono or poly(ADP-ribose) levels *in vivo*. The major problem here is the very low levels observed *in vivo* thus making study of individual proteins impractical. A further complication is that if ADP-ribosylation performs two or more functions as seems likely then, changes in the ADP-ribosylation of a single protein or small class of protein may not be detected.

The most fruitful long term approach must surely be the identification of the proteins which ADP-ribosylated in the cells followed by elucidation of their biological role. This would involve development of isolation procedures under non-denaturing conditions and is clearly a mamoth task.

The final approach used has been the treatment of cells with inhibitors of poly(ADP-ribose) synthetase with subsequent examination of cellular dysfunction. The major problem encountered is the lack of physiological specificity

of inhibitors used to date. The inhibitors available for *in vivo* studies are restricted to nicotinamide and 5-methylnicotinamide, thymidine and its analogues and the methylated xanthines. All of these compounds affect cellular processes other than ADP-ribosylation. Nicotinamides act as substrates for NMN pyrophosphorylase and may deplete the cellular phosphoribosyl pyrophosphate pool as suggested by Hilz (232). Thymidine is known to inhibit DNA synthesis by depletion of the dCTP pool (233). Methylated xanthines affect cyclic AMP phosphodiesterase (9). All three groups of compounds have also been shown to inhibit the NAD glycohydrolase activity in rabbit reticulocytes (234,235) and more recently 5-methlnicotinamide and theophylline has been found to inhibit NAD glycohydrolase in LS1210 cells (236). Shall's group have attempted to circumvent the problem of the lack of specificity of these inhibitors by showing that all three groups of inhibitors inhibit DNA repair (179). This, however, is also dangerous because caffeine, a methylated xanthine, inhibits DNA by a different mechanism than the other inhibitors (193).

It was for these reasons that the present work of synthesizing and screening new inhibitors of poly(ADP-ribose) synthetase was undertaken. The following sections describe the synthesis of such compounds, their potency of inhibition of poly(ADP-ribose) synthesis *in vitro*, a limited study of their physiological specificity and the effect on LS1210 cells.

SECTION 2

ORGANIC SYNTHESES

2.1. Purification of 3-aminobenzamide

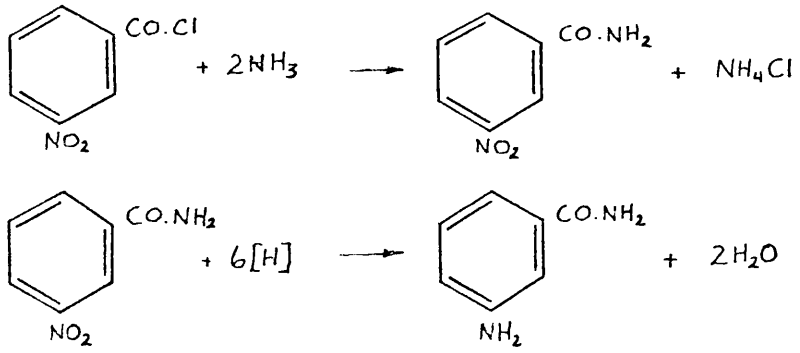
Commercial 3-aminobenzamide was obtained from Fluka Ltd. It was a purplish powder with a melting point of 101-4°C. Pure 3-aminobenzamide is known to be either yellow needles with a melting point of 80°C when recrystallized from water or colourless needles with a melting point of 114°C when recrystallized from absolute ethanol (237). In an attempt to purify the commercial 3-aminobenzamide, recrystallization from water was attempted. This gave a pinkish purple product with a melting point of 106°C.

Recrystallization was attempted from various organic solvents such as methanol, ethanol, acetone and diethylether. The cleanest product was obtained by extraction of the commercial preparation with anhydrous benzene as follows:- 20-25g of finely powdered 3-aminobenzamide was placed in a GF/C thimble (Whatman). This was continuously extracted with boiling benzene (200ml) in a Soxhlet extractor. After a time, the solvent in the lower vessel turned yellow and eventually solid appeared. After 1-2 hours the benzene was allowed to cool and the solid collected by filtration. The filtrate was then reused for a further 2 hours extraction and again the solid was collected by filtration. The combined product was dried under vacuum overnight. The product was yellowish orange with a melting point of 106°C.

The low yield (10-20%) of product made this process an extremely time-consuming and expensive one. An attempt was therefore made to synthesize 3-aminobenzamide.

2.2. Synthesis of 3-aminobenzamide

This was achieved in two steps using 3-nitrobenzoyl chloride (Aldrich) as starting material. The acid chloride was converted to the amide (238) and then the nitro group was reduced.



(a) 3-nitrobenzamide

5g of ammonium acetate (BDH, analar) was suspended in 70ml dry acetone (dried over anhydrous sodium sulphate) and 5g of finely powdered 3-nitrobenzoyl chloride was added in small portions. The mixture was left stirring for 1 hour at room temperature after which time the precipitated ammonium chloride was removed by filtration. The filtrate was then evaporated to dryness under reduced pressure. The solid was dissolved in a minimum quantity of boiling water, decolourized with activated charcoal and the 3-nitrobenzamide crystallized out upon cooling. The product was creamy white needles with a melting point of 143°C. The yield was 74%. A small portion of the sample was applied to a PEI-cellulose t.l.c. sheet and developed in n-butanol; methanol; water; ammonia (60;20;20;1; by vol). After running to approximately 10cm above the origin, the plate was dried and the

products visualized under u.v. light. All the u.v. absorbing material was at the solvent front; none could be detected with an Rf of 0.6 which is the position at which 3-nitrobenzoic acid migrates.

(b) Reduction of 3-nitrobenzamide

5g 3-nitrobenzamide was dissolved in 200 ml of absolute ethanol and the solution was placed in a pear-shaped flask. 200mg of 10%(w/w) palladium on charcoal was added and the mixture stirred. Hydrogen gas was slowly bubbled through the suspension (this step was carried out in a fume cupboard). At various intervals, a small aliquot was removed, applied to a 2cm x 2cm piece of PEI-cellulose and dried. The completion of the reaction was signified by the conversion of the material from u.v. absorbing to a bright blue fluorescent spot when visualized under u.v. light. When complete, the reaction mixture was filtered to remove the catalyst and the solvent was removed by distillation under reduced pressure. The product was creamy white and had a melting point of 110^{o}C (3.9g, 95% yield).

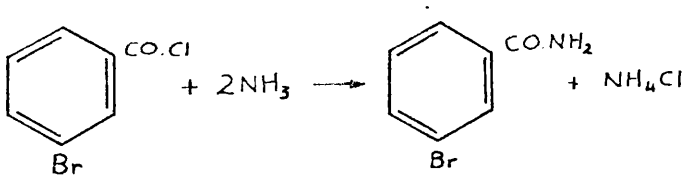
The purity of the material was monitored by t.l.c. on PEI-cellulose using the butanol system (see above). The plate was dried and visualized under u.v. light. A single bright blue fluorescent spot with an Rf of 0.54 was observed. No u.v. absorbing material was observed (ie nitrobenzamide). No fluorescent material with an Rf of 0.45(ie 3-aminobenzoic acid) was observed.

A spectrum of the material dissolved in ethanol was obtained and this is shown in Fig. 2. The characteristics are a peak at 311nm (log ε = 3.26) and a large peak at 218nm (log ε = 4.34) with a shoulder at 240nm (log ε = 3.85). This is in good agreement with the values quoted in the CRC Handbook of Spectroscopy, namely a peak at 310nm (log ε = 3.3) and a shoulder at 240nm (log ε = 3.8).

A summary of the analytical data for the various 3-aminobenzamide preparations is given in Table 2.

2.3. Synthesis of 3-bromobenzamide

3-Bromobenzamide was synthesized using a similar method to that used for 3-nitrobenzamide (2.2.a).



5g of 3-bromobenzoyl chloride (Koch-Light Lab.) was added dropwise to a stirred suspension of 5g ammonium acetate in 70ml dry acetone. After 1 hour, the mixture was filtered and the solvent was removed under reduced pressure. The solid was recrystallized from boiling water and the final product was obtained as silky white needles (3.74g) with a melting point of 154-6^{o}C (155^{o}C from CRC Handbook of Physics & Chemistry, 59th edition).

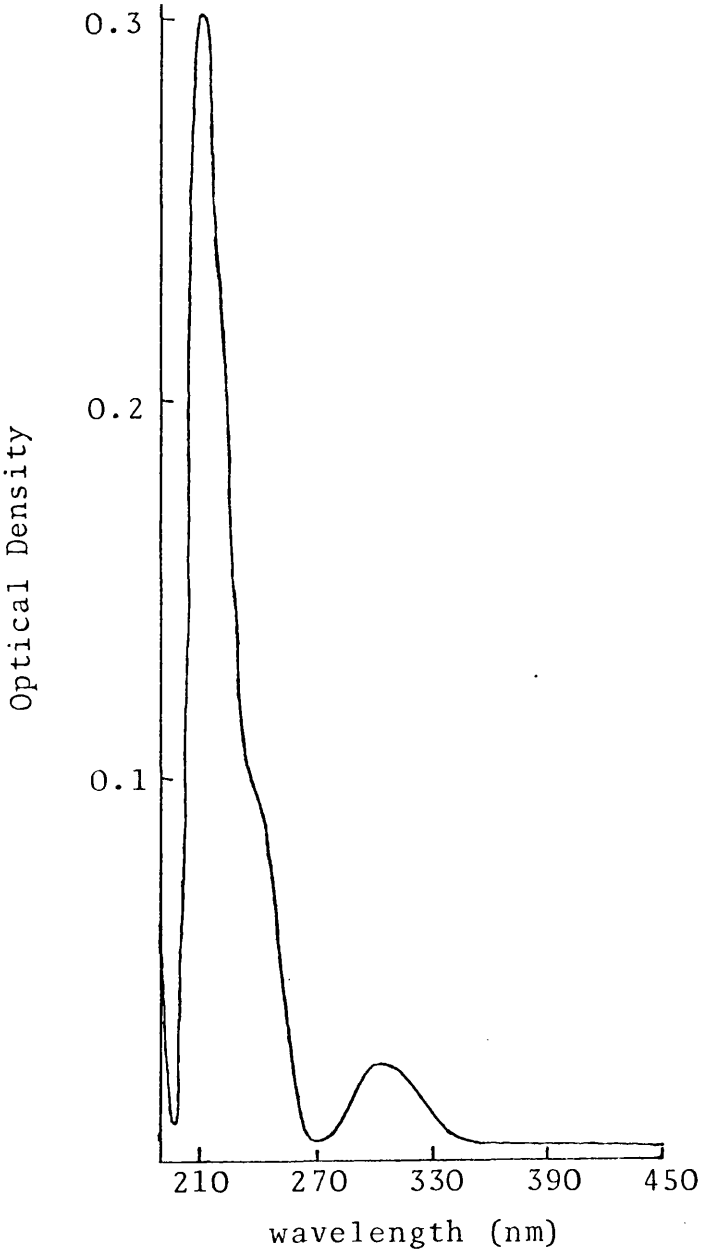


Figure 2 U.V. spectrum of 16μM 3-aminobenzamide.

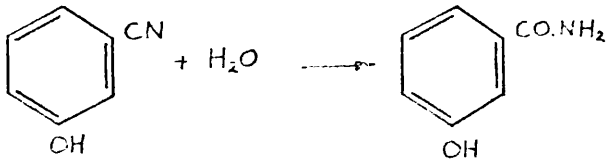
Table 2 Analytical details for various 3-aminobenzamide preparations.

Source	Appearance	M. Pt.	U.V. Spectra
Fluka	purple	101-4°C	-
Recrystallized from benzene	yellow	106°C	-
Synthesized	cream	110°C	peak 311nm sh. 240nm
Literature	yellow colourless	80°C 114°C	peak 310nm sh. 240nm

2.4. Synthesis of 3-hydroxybenzamide

In view of the low yield of 3-hydroxybenzamide obtained by the ammoniolysis of 3-hydroxybenzoic acid ethyl ester (239), an attempt was made to synthesize 3-hydroxybenzamide by the limited hydrolysis of 3-hydroxybenzonitrile using slightly alkaline hydrogen peroxide. This was first used by Radziszewski in 1885 (240).

It has since been used for the preparation of various aromatic amides (241,242) and, in one report, it was used to synthesize 3-hydroxybenzamide although no practical details were given (243).



In order to follow the reaction, a method was developed to detect the various reaction products after resolution by t.l.c. When 3-hydroxybenzoic acid or 3-hydroxybenzonitrile were applied to a t.l.c. plate and viewed under u.v. light, no spots could be observed. 3-hydroxybenzoic acid (244-246) and 3-hydroxybenzamide (246) have been visualized after paper chromatography by spraying with diazotized aromatic amines. 3-hydroxybenzoic acid, however, is known to be fluorescent at high pH (247). It was found that both 3-hydroxybenzoic acid and 3-hydroxybenzonitrile gave fluorescent spots, which faded after a time, on exposure to ammonia vapour.

The method used was as follows:-

5g 3-hydroxybenzonitrile (3-cyanophenol, Aldrich) was suspended in 45ml water and 4M NaOH was added until the pH was approximately 8.5. The suspension was warmed to 50°C and 10ml 100 vol hydrogen peroxide was added. After 90 minutes, the mixture was adjusted to pH 6 with hydrochloric acid and then lyophilized. The solid was extracted with 50ml chloroform to remove unreacted 3-hydroxybenzonitrile and then the hydroxybenzamide was extracted with two 50ml portions of diethylether. After removal of the ether by rotary evaporation the solid was recrystallized from boiling water. The yield of fine white crystals [melting point 165°C compared to 169°C (251)] was 2.19g (38%). When analysed by t.l.c. in the butanol system the product gave a single blue fluorescent spot with an Rf of 0.81. The nitrile ran with the solvent front and the acid had an Rf of 0.46.

The u.v. spectra of the final product in ethanol is shown in Fig. 3. The major characteristics are in agreement with those previously reported, ie a shoulder at 230nm and a peak at 295nm (CRC Handbook of Spectroscopy, vol. 2).

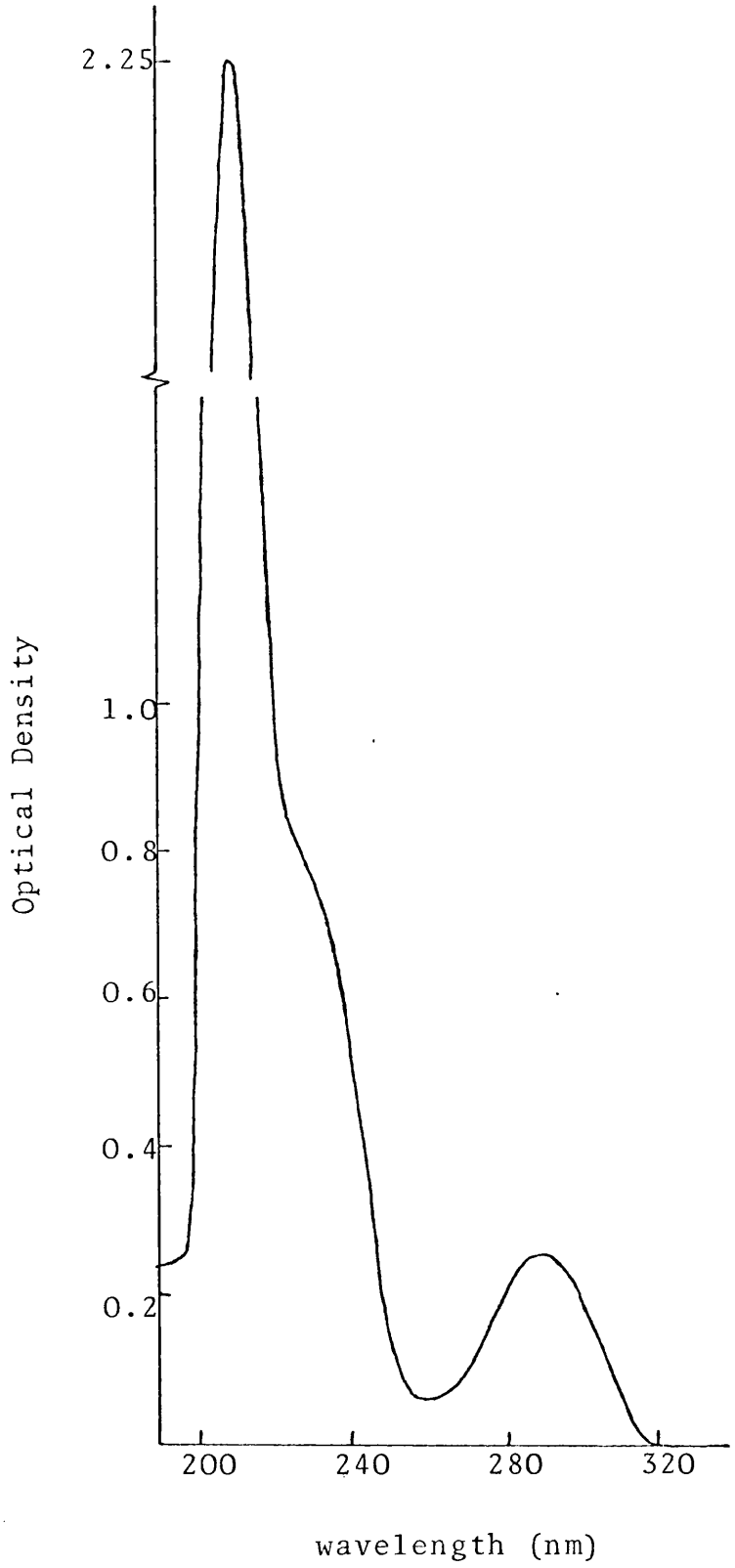
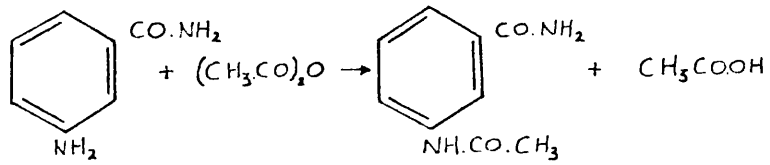


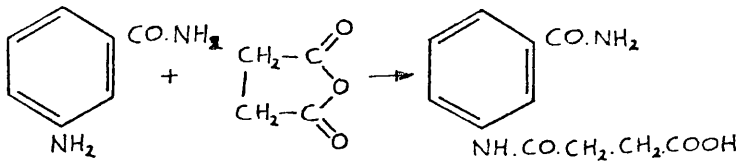
Figure 3 U.V. spectrum of 100μM 3-hydroxybenzamide.

2.5. Synthesis of 3-acetylaminobenzamide



0.5g 3-aminobenzamide (Section 2.2) was dissolved in 15ml acetone and 5ml acetic anhydride was added. After about 30 seconds, a dense white precipitate appeared. The reaction was allowed to continue for about 10 minutes. The precipitate was collected by filtration, washed twice with acetone and recrystallized from water. 3-acetylaminobenzamide was obtained as long white needles with a melting point of 214-217°C (216°C, Ref. 242). When a small sample was examined under u.v. light no fluorescence could be detected indicating the absence of free 3-aminobenzamide. The final yield was 0.58g(84%).

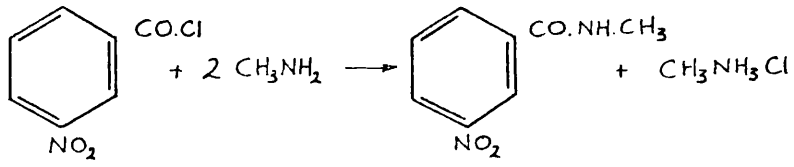
2.6. Synthesis of 3-succinylaminobenzamide



1.36g 3-aminobenzamide (Section 2.5) was dissolved in 10ml dry pyridine and to this was added 1.1g succinic anhydride. After 12 hours at room temperature, 10ml of water was added and the mixture left for a further hour. The solvent was removed by rotary evaporation and then

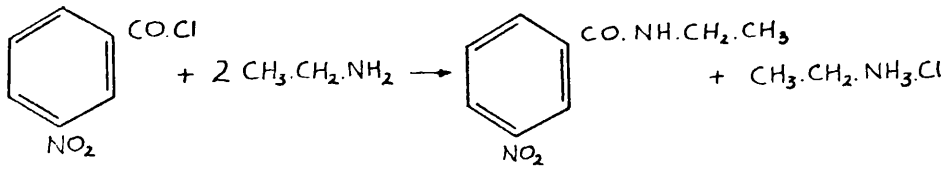
10ml of water was added which was also removed by evaporation. The final product was recrystallized from water as small white needles with a melting point of 199-201°C (203-205°C, Ref. 242).

2.7. Synthesis of 3-nitrobenzamidomethane



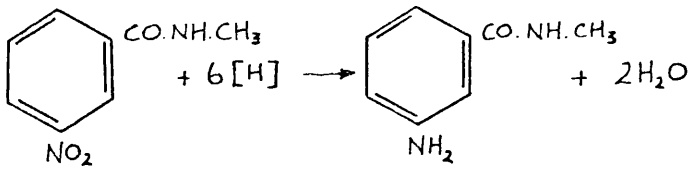
9.25g of powdered 3-nitrobenzoyl chloride (Aldrich) was added gradually to 25ml of a 33% (w/v) solution of methylamine in methanol (BDH). After stirring for 1 hour, the solvent and excess methylamine were removed under reduced pressure. The solid residue was extracted once with 50ml diethyl ether to remove 3-nitrobenzoic acid and its methyl ester. The remaining solid recrystallized from boiling water. The product (4.9g, 54% yield) crystallized as fine yellow needles with a melting point of 172-3°C (174°C from Belstein). After chromatography on PEI-cellulose in the butanol system, a large dark spot was observed at the solvent front with a faint trace migrating as 3-nitrobenzoic acid (Rf = 0.62) when the dried chromatogram was viewed under u.v. light.

2.8. Synthesis of 3-nitrobenzamidoethane



1.85g of 3-nitrobenzoylchloride was dissolved in 25ml dry chloroform and the solution placed on ice. 2.6ml of anhydrous ethylamine was added slowly with frequent swirling. The reaction was allowed to proceed for 30 minutes. The solvent and excess ethylamine were blown off under a stream of dry air. 10ml of water was added and the mixture was stirred vigourously for 10 minutes. The yellow solid was collected by filtration and washed with cold water. The product was recrystallized from hot water and separated out as long yellow needles with a melting point of 111°C. The yield was 42%. T.l.c. analysis revealed a large spot at the solvent front with a trace of 3-nitrobenzoic acid.

2.9. Synthesis of 3-aminobenzamidomethane



0.5g 3-nitrobenzamidomethane was dissolved in 50ml absolute ethanol and 50mg 10% palladium on charcoal was added. Hydrogen was then bubbled through the mixture and the extent of reduction was monitored as described

previously (Section 2.2). The catalyst was removed by filtration and the solvent by rotary evaporation. The product (94%) yield was white and gave a single blue fluorescent spot at the solvent front when analysed by t.l.c. The melting point was 115-117^{o}C.

SECTION 3

SCREENING OF POTENTIAL INHIBITORS OF POLY(ADP-RIBOSE) SYNTHETASE

3.1. Introduction

The criteria for the use of a compound as a physiologically specific inhibitor of an enzyme are as follows:-

(a) the compound should be soluble in water;

(b) it should be able to enter the cell;

(c) it should not be metabolized rapidly, if at all;

(d) the compound should inhibit the target enzyme and should, ideally, have no effect on other cellular processes.

All the previously known inhibitors of poly(ADP-ribose) synthetase can be excluded on one or more of these conditions. The main problem with the nicotinamides is that, because they possess the pyridine ring nitrogen atom, they interfere with NAD synthesis (see Section 4.3). Benzamide, which lacks the pyridine nitrogen, would thus be expected to have little or no effect. As stated previously, benzamide has been reported to be a good inhibitor of poly(ADP-ribose) synthetase although no data was given (98). The major problem with the use of benzamide as a physiological inhibitor is its very low solubility in water. The most useful approach was to examine benzamides which had been substituted in the 3-position for their ability to inhibit poly(ADP-ribose) synthetase.

For initial screening, a 0.5M NaCl extract from pig thymus nuclei was used as a source of poly(ADP-ribose) synthetase because of its high activity and relative stability.

3.2. Methods

3.2.1 Isolation of pig thymus nuclei and extraction of poly(ADP-ribose) synthetase with 0.5M NaCl

Thymii of freshly slaughtered pigs were obtained from Bowyers Ltd, Trowbridge, Wilts. The connective tissue was removed and they were frozen at -20°C until use.

10-15g of frozen thymus was cut into thin slices and placed into 200ml 0.32M sucrose; 3mM $MgCl_2$ and 10ml 0.25M Tris. HCl pH 7.5 which had been cooled to 0.4°C (all subsequent steps were performed at this temperature). This was homogenized in a Sorvall "Omnimixer" at setting 2 for 30 seconds three times, with 30 seconds between each burst. The homogenate was filtered through muslin to remove connective tissue and then centrifuged at 2,500 rpm for 10 minutes in an MSE bench centrifuge. The pellet was re-suspended in 75ml 2.2M sucrose; 3mM $MgCl_2$ and 2.5ml 0.25M Tris. HCl pH 7.5. This was centrifuged at 22,000 rpm for 1 hour in a Beckman SW 27.1 rotor. The pellet was then gently resuspended in 20ml 0.5M NaCl; 0.1M methanolamine HCl pH 8.2; 10mM $MgCl_2$; 5mM DTT (dithiothreitol) and left on ice for 1 hour. This was then centrifuged at 50,000 rpm for 2 hours in a Beckman SW 50.1 rotor. The supernatant was removed and frozen in 2ml aliquots until use.

3.2.2 Poly(ADP-ribose) synthetase assay

Poly(ADP-ribose) synthetase was assayed by the incorporation of [^{3}H-ade]-NAD into acid-insoluble material. The reaction mixture contained 100mM triethanolamine HCl pH 8.2; 10mM $MgCl_2$; 2mM DTT; 50µM [^{3}H-ade]-NAD (200µCi/mmole) in 180µl. After equilibration at 26°C, 20µl of the 0.5M NaCl extract was added. The acid-insoluble radioactivity was determined by placing 20µl of the reaction mixture onto a paper filter disc (Whatman 3MM, 2.4cm diameter), which had been presoaked in 20% (w/v) TCA in ether and dried. The disc was placed into 20% (w/v) TCA (in water) and left on ice for at least 30 minutes. Discs were then washed batchwise four times in 20% TCA allowing 5 minutes for each wash. After an ethanol wash and a diethyl ether wash (to remove the TCA), the disc was allowed to dry at room temperature. The radioactivity was determined by scintillation counting in 2ml 0.5% (w/v) PPO in toluene.

3.2.3 Effect of 3-aminobenzamide on the stability of the ADP-ribose - protein linkage

A 1ml reaction mixture was set up and the reaction was allowed to proceed for 5 minutes. To this was added 8M urea to a final concentration of 4M or urea and 3-aminobenzoic acid to a final concentration of 4M and 2mM respectively. Aliquots of the mixture were then analysed for acid-insoluble radioactivity at various times.

3.3. Results and Discussion

3.3.1 ADP-ribosylation and the effect of benzamides

Fig. 4 shows the time course of incorporation of [^{3}H]-NAD into acid-insoluble material. It rapidly loses linearity for a number of reasons. These include inactivation of the enzyme (248), lability of the protein-ADP-ribose linkage, and degradation of the product by poly(ADP-ribose) glycohydrolase and proteolysis. For screening various compounds, a time of 5 minutes was chosen. The Km for NAD was determined and found to be 51μM (Fig. 5). This was the concentration of NAD used in all subsequent synthetase assays unless otherwise stated. Under these conditions, the two most commonly used inhibitors of poly(ADP-ribose) synthetase, nicotinamide and thymidine, decreased incorporation by 63% and 48% respectively. The observation of Shall and co-workers (98) that benzamide was a good inhibitor was confirmed (Table 3). Terada *et al* (215) have recently reported that benzamide was a more potent inhibitor than nicotinamide when using a partially purified enzyme preparation from calf thymus and isolated nuclei from Friend cells.

The effect of substitution of the benzene ring was studied using the three isomers of aminobenzamide. As can be seen in Table 3, 3-aminobenzamide was much more potent than either 2-aminobenzamide or 4-aminobenzamide. This is probably due to steric hindrance because Preiss *et al* (96) found that, with the exception of 5-methyl nicotinamide,

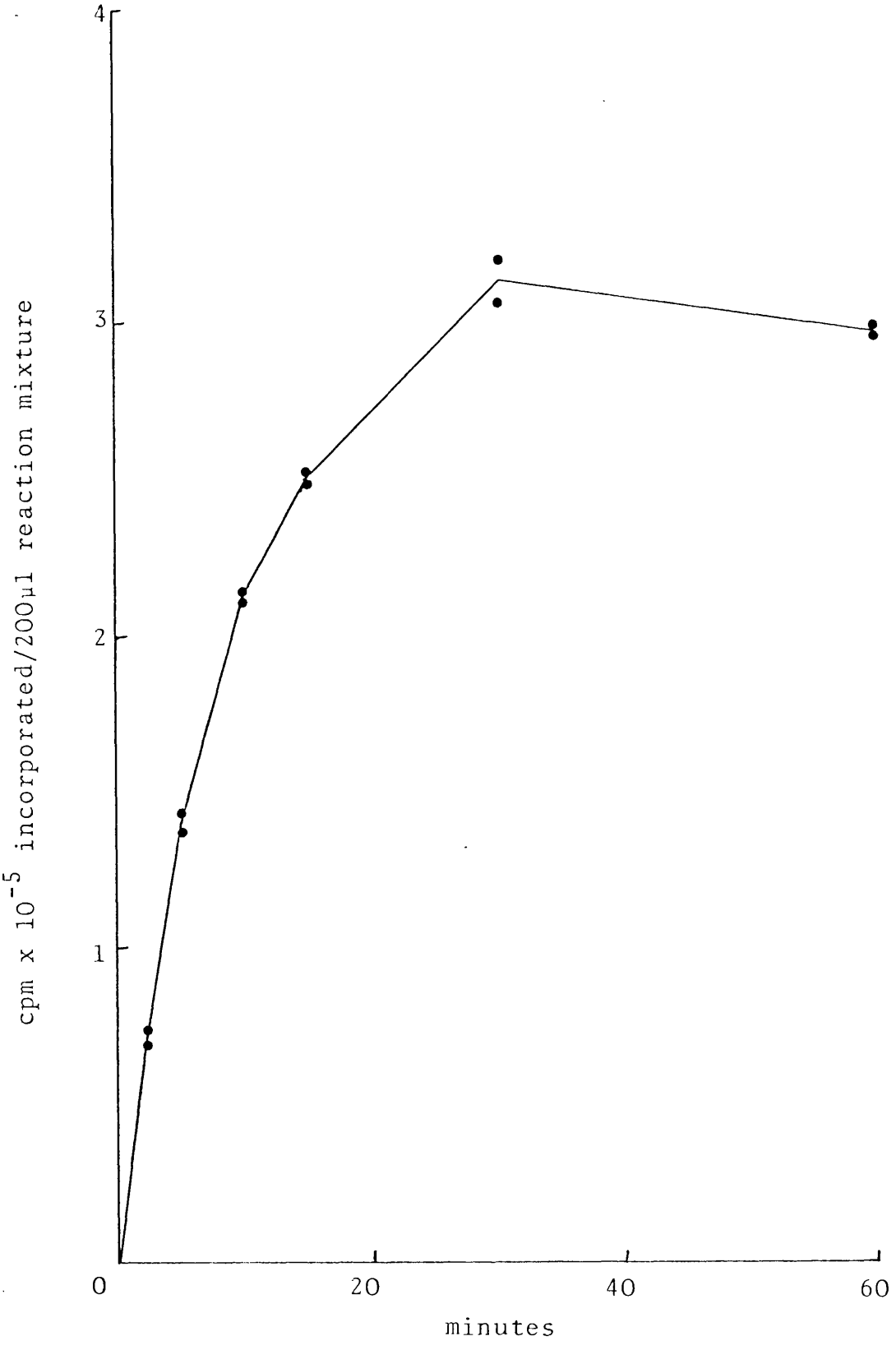


Figure 4 The time course of incorporation of ^{3}H-NAD into acid-insoluble material by a pig thymus nuclear extract.

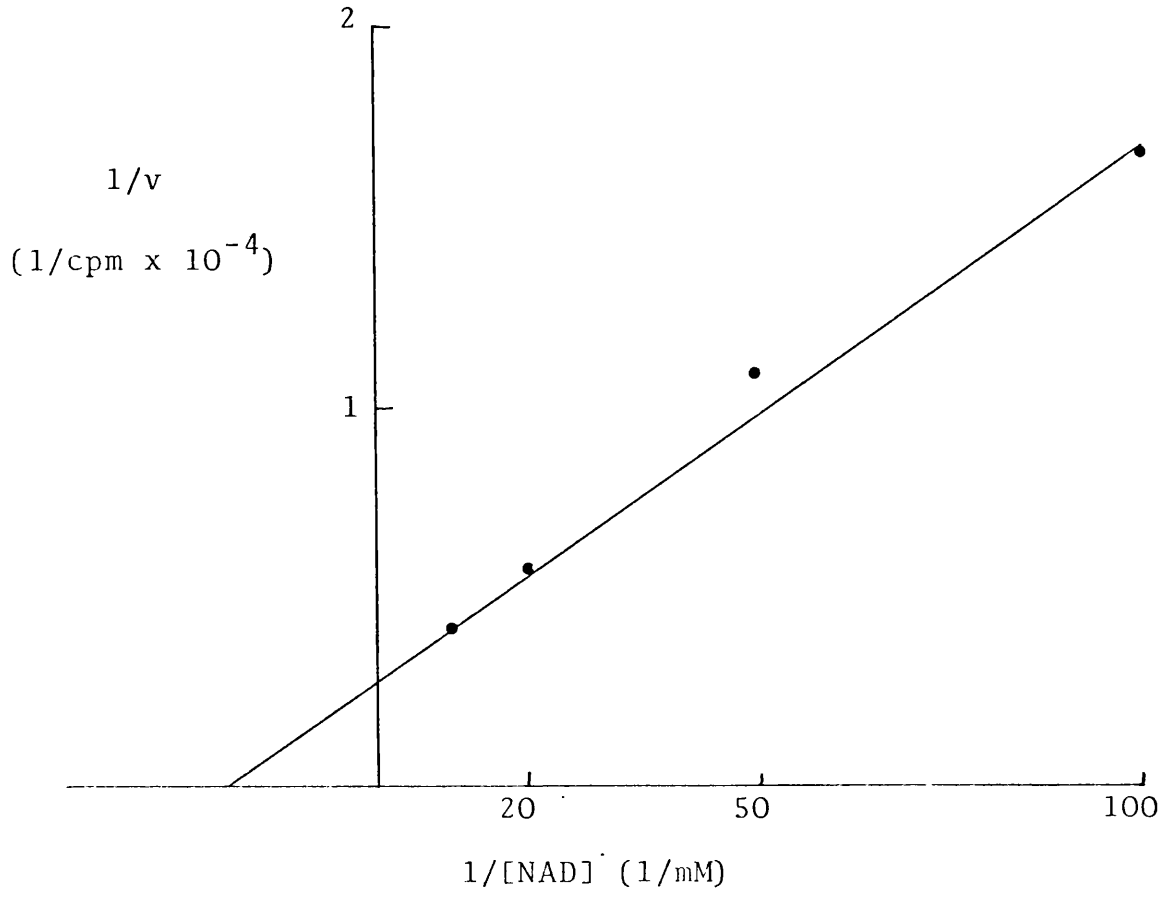


Figure 5 Km determination for NAD.

Table 3 The effect of benzamides on poly(ADP-ribose) synthetase from pig thymus

The figures in parentheses represent the percentage inhibition.

Addition (50μM)	Incorporation of NAD into acid-insoluble material (nmol/min/mg protein)	Approx. Ki (μM)
Control	2.40	-
Benzamide	0.09 (96)	0.99
2-Aminobenzamide	1.98 (17)	120
3-Aminobenzamide	0.23 (90)	2.6
4-Aminobenzamide	1.89 (21)	94
3-Acetylaminobenzamide	0.06 (98)	0.43
3-Bromobenzamide	0.12 (95)	1.4
3-Hydroxybenzamide	0.09 (96)	1.0
3-Methoxybenzamide	0.06 (98)	0.61
3-Nitrobenzamide	0.67 (71)	9.8
3-Succinylaminobenzamide	0.22 (91)	2.56

ring substitution of nicotinamide drastically reduced the inhibitory potency of such analogues. It is interesting that nicotinamide and its isomer, picolinamide, were equally good inhibitors of ADP-ribosylation in pancreatic nuclei (249).

The mode of inhibition for 3-aminobenzamide was determined. Although the data is displayed as a Lineweaver-Burke plot, Vmax and Km (app) values were determined using the direct linear plot (250). Fig. 6 shows 3-aminobenzamide was a competitive inhibitor with a Ki of 1.8μM.

A variety of other benzamides with different groups at the 3 position were examined to determine if more powerful inhibitors could be found. Table 3 shows the results obtained. All the benzamides substituted in the 3 position were more inhibitory than either nicotinamide or thymidine. 3-methoxybenzamide was found to be a competitive inhibitor with a Ki of 1.2μM (Fig. 7). Assuming all the other inhibitors are also competitive, their Ki values can be estimated by substituting reaction rates into the following equation

$$Ki = \frac{\frac{vi}{v} \cdot [i]}{2(1 - \frac{vi}{v})}$$

where vi = rate in presence of inhibitor, v = rate of control and [i] = inhibitor concentration. These values are included in Table 3.

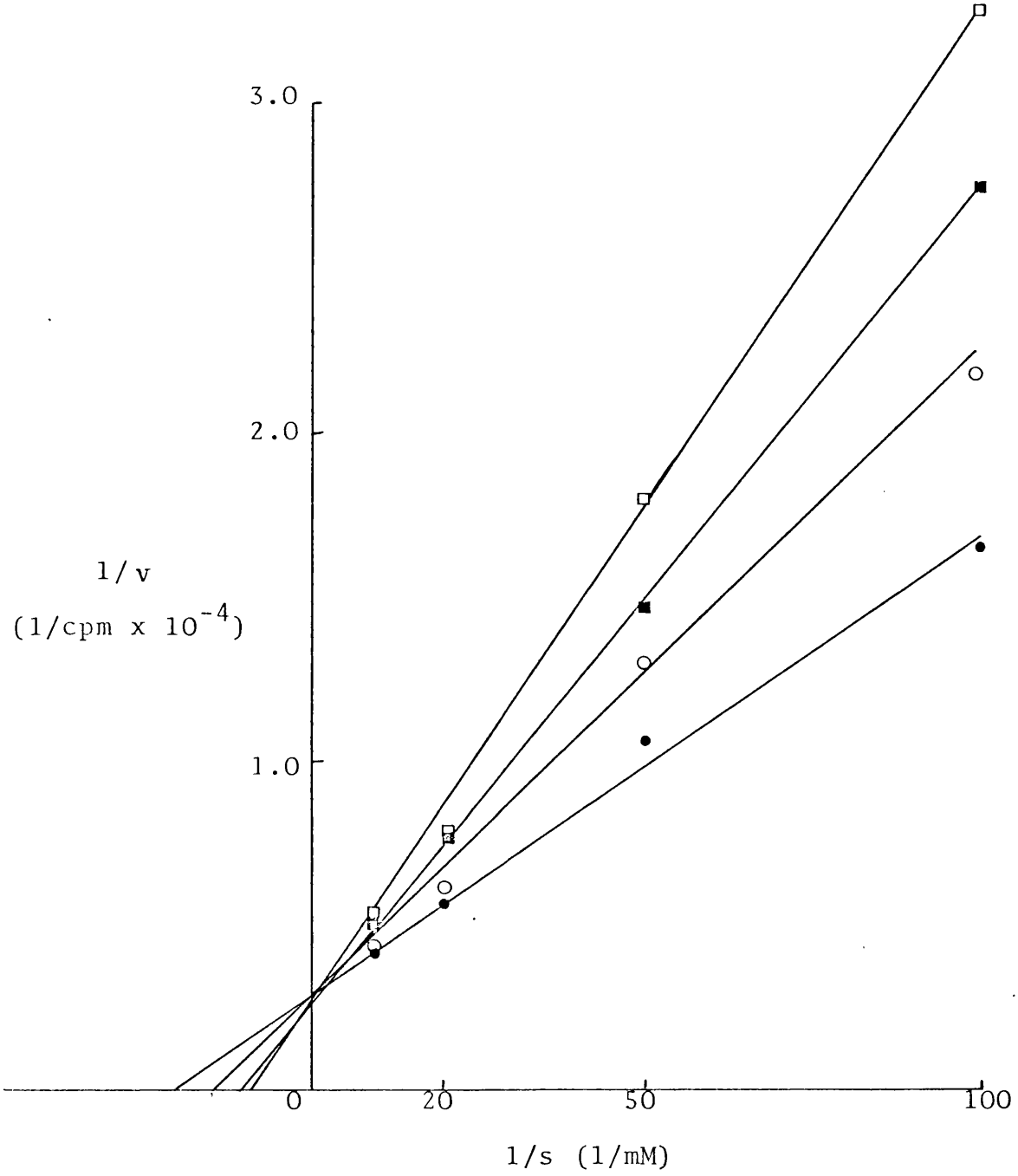


Figure 6 Ki determination for 3-aminobenzamide.

●, control; ○, 0.5μM 3AB; ■, 1μM 3AB; □, 2μM 3AB

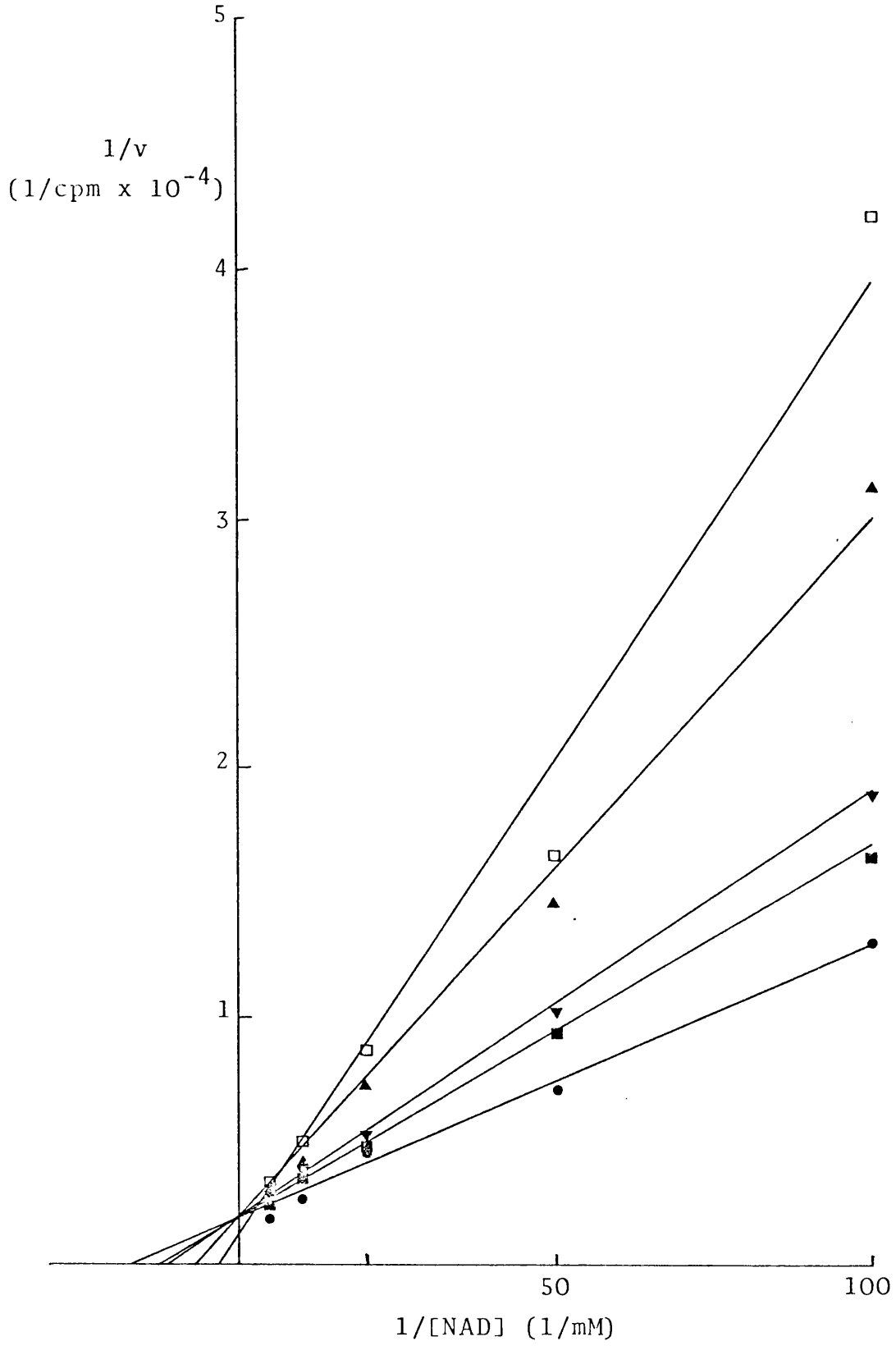


Figure 7 Ki determination for 3-methoxybenzamide.

Concentration of 3-methoxybenzamide: •, 0;

■, 0.5μM; ▼, 1μM; ▲, 2μM; □, 2.5μM

The difference in the inhibition of poly(ADP-ribose) synthetase elicited by the various benzamides and nicotinamide is not easily understood. Obviously the size of the substituent group is unimportant because 3-succinylaminobenzamide and 3-acetylaminobenzamide are as good if not better inhibitors than 3-aminobenzamide. Similarly benzamide and nicotinamide would be expected to be equally good inhibitors.

The binding of 3-substituted benzamide to the substrate site of horse liver alcohol dehydrogenase has been reported to be primarily controlled by the electron density of the carbonyl group (251). If, however, the inhibition of poly(ADP-ribose) synthetase by benzamides is compared with the Hammet constant (σ) of the substituent group (252), no obvious correlation is apparent (Table 4). Although 3-nitrobenzamide, the worst inhibitor of the benzamides tested, had a higher σ value than all the others, 3-acetylaminobenzamide has a higher σ constant than 3-aminobenzamide and is also a better inhibitor.

The overall hydrophobicity of the various benzamides would explain the greater inhibition by 3-acetylaminobenzamide compared to 3-aminobenzamide and also the difference between 3-methoxybenzamide and 3-hydroxybenzamide. Hydrophobicity would also explain why the benzamides as a group are all better inhibitors than nicotinamide. 3-nitrobenzamide, however, is more hydrophobic than either 3-aminobenzamide or 3-hydroxybenzamide as judged by their Rf values

Table 4 Hammet constants and inhibitory potency of benzamides

Hammet constants are from McDaniel & Brown (252).

Compound	Approx. Ki (μM)	σ value
3-Acetylaminobenzamide	0.43	0.21
3-Methoxybenzamide	0.61	0.115
Benzamide	0.99	0
3-Hydroxybenzamide	1.0	0.121
3-Bromobenzamide	1.4	0.391
3-Aminobenzamide	2.6	-0.16
3-Nitrobenzamide	9.8	0.710

Table 5 Rf values of 3-aminobenzamide, 3-hydroxybenzamide and 3-nitrobenzamide

Samples were applied to PEI-cellulose and chromatographed in n-butanol; methanol; H_2O; conc.NH_4OH.

	Rf
3-Nitrobenzamide	1.0
3-Hydroxybenzamide	0.81
3-Aminobenzamide	0.54

when chromatographed on PEI-cellulose in butanol/methanol/water/ammonia (Table 5) and is a worse inhibitor. In view of the much higher Hammet constant for 3-nitrobenzamide as compared to the other benzamides tested, the greater polarity of the carbonyl group resulting from electron withdrawal from the amide group may destabilize the inhibitor-enzyme complex more than the hydrophobicity of 3-nitrobenzamide stabilizes it. Electron withdrawal by the ring nitrogen may also contribute to the lower inhibition by nicotinamide.

The above data suggest that the binding of benzamides to poly(ADP-ribose) synthetase is controlled both by the hydrophobicity of the ligand and the electron density of the amide group. In view of the structural similarity between benzamide and nicotinamide and the competitive inhibition of the enzyme by benzamides, it is tempting to suggest that the benzamides are binding to the nicotinamide portion of the NAD binding site. The presence of a hydrophobic region would favour the rapid dissociation of nicotinamide after cleavage of the N-glycosidic linkage. Such a hydrophobic region could also contribute to the hydrolysis of NAD by repelling the positively charged quaternary nitrogen of NAD and thus distorting the N-glycosidic linkage. The findings that nicotinamide analogues with a quaternary nitrogen such as N^1-methyl-nicotinamide (96,215) and NMN (96) are extremely poor inhibitors of the enzyme is consistent with such a possibility.

The possible use of benzamides as affinity ligands for purification of poly(ADP-ribose) synthetase prompted a search for means of immobilizing benzamides with retention of inhibition. All the benzamides could be linked through the amide group (ie $X.C_6H_4.CO.NH.CH_2.$). Four such compounds were tested for their ability to inhibit poly(ADP-ribose) synthetase and the results are shown in Table 6. None of the compounds tested inhibited the enzyme. Two corresponding nicotinamide analogues, N'-ethylnicotinamide (96) and N'-methylnicotinamide (215), were also found to have no effect on ADP-ribosylation. Clearly this method of immobilization is unsuitable for preparation of an affinity ligand. The aromatic amine of 3-aminobenzamide is a reactive group and could be used for immobilization via a secondary amine, amide or azo linkage. The second possibility viz. an amide linkage is a promising one because 3-acetylaminobenzamide and 3-succinylaminobenzamide are both extremely good inhibitors (Table 3).

On the basis of the inhibition elicited by the various benzamides, the choice of one suitable for use as a probe for the function of ADP-ribosylation in vivo is a difficult one. On the basis of the data presented above, the best inhibitors are the more hydrophobic and consequently less soluble. The synthesis of a more soluble derivative of 3-acetylaminobenzamide ie 3-[hydroxyacetyl]-aminobenzamide was attempted by two means. The first involved the condensation of glycollic acid and 3-aminobenzamide using dicyclohexylcarbodiimide. Problems were encountered with

Table 6 Effect of N'-substituted benzamides on poly(ADP-ribose) synthetase activity

Addition (50μM)	Incorporation of NAD into acid-insoluble material (nmol/min/mg protein)
Control	2.40
3-Aminobenzamidomethane	2.38
3-Nitrobenzamidomethane	2.45
3-Nitrobenzamidoethane	2.37

the side reactions produced by the presence of the highly reactive α-hydroxyl group of glycollic acid and the isolation of the desired product. This method was abandoned and the synthesis was attempted via the bromoacetyl derivative. This method was also abandoned because of the complexity of the products from the reaction between bromoacetyl chloride and 3-aminobenzamide.

3.3.2 The effect of acetophenones on ADP-ribosylation

3-Acetyl pyridine, a close analogue of nicotinamide, has been reported to inhibit poly(ADP-ribose) synthetase from rat liver (253) and chick embryo limb buds (204) whereas the enzymes from HeLa cells (96), calf thymus and Friend cells (215) appear to be insensitive. Table 7 shows that under the present conditions, no inhibition of the pig thymus enzyme by 3-acetyl pyridine was observed. In contrast, most of the acetophenones were good inhibitors of ADP-ribosylation. The nature of the substituent group affects the inhibition produced by the various compounds. A fairly close correlation is seen between the inhibition by a particular acetophenone and the corresponding benzamide; the 3-methoxy compounds are the best inhibitors, the 3-nitro compounds are the worst and the 3-amino compounds are worse than the unsubstituted compounds. In view of this correlation, it is highly probable that the benzamides and acetophenones are binding at the same site on the enzyme and suggests that the presence of the amide nitrogen is not therefore a prerequisite for inhibition of the enzyme.

Table 7 Effect of acetophenones on poly(ADP-ribose) synthetase activity

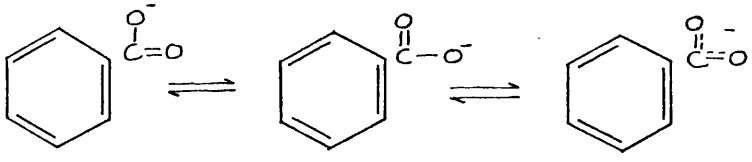
The figures in parentheses are the percentage inhibition.

Addition (50μM)	Incorporation of NAD into acid-insoluble material (nmol/min/mg protein)
Control	2.63
3-Acetylpyridine	2.70 (0)
Acetophenone	1.68 (36)
3-Aminoacetophenone	1.84 (30)
3-Bromoacetophenone	1.52 (42)
3-Hydroxyacetophenone	1.39 (47)
3-Methoxyacetophenone	1.15 (56)
3-Nitroacetophenone	2.55 (3)
Propiophenone	2.47 (6)
α,α,α, Trifluoroacetophenone	2.44 (7)

The lower inhibition produced by the acetophenones cannot be explained by their hydrophobicity because they are less soluble than the benzamides. The acetyl group differs from the amide in two major aspects: it is non-planar and non-polar. Using acetophenones, it is not possible to distinguish which of these properties is the more important. The lack of inhibition by propiophenone and α,α,α,-trifluoro-acetophenone suggests that the enzyme has a rigorous steric requirement in the carboxamide/carbonyl region of the binding site.

3.3.3 The effect of benzoic acids on ADP-ribosylation

Nicotinic acid has not been reported to inhibit poly(ADP-ribose) synthetase from any source. A number of benzoic acids were screened. Unlike the acetophenones the benzoic acids are both planar and very polar. At the pH of the assay (8.2), they are ionized. The negative charge is delocalized.



The results obtained are shown in Table 8. With the exception of 3-aminobenzoic acid, none of the benzoic acids (nor nicotinic acid) are inhibitory under the present conditions. The purity of the commercial 3-aminobenzoic acid used (Aldrich Chem. Co. Gold Label, 99% pure) was checked for possible contamination by 3-aminobenzamide.

Table 8 The effect of benzoic acids on poly(ADP-ribose) synthetase activity

Addition (50μM)	Incorporation of NAD into acid-insoluble material (nmol/min/mg protein)
Control	2.40
Nicotinic acid	2.43
2-Aminobenzoic acid	2.59
3-Aminobenzoic acid	2.16
4-Aminobenzoic acid	2.47
3-Hydroxybenzoic acid	2.38
3-Nitrobenzoic acid	2.45

When analysed by t.l.c. no fluorescent material migrated with 3-aminobenzamide. In addition, a portion of 3-aminobenzoic acid was treated with 6M hydrochloric acid for 48 hours at room temperature (under these conditions, 3-aminobenzamide was completely hydrolysed) and tested for its ability to inhibit the enzyme; no decrease in inhibition was observed. Thus the inhibition of poly(ADP-ribose) synthetase is not an artefact. Durkacz et al have recently reported that 3-aminobenzoic acid was not an inhibitor of ADP-ribosylation in permeabilized L1210 cells (195).

It has been shown that 3-aminobenzoic acid inhibits NAD glycohydrolase from pig spleen (254) and the suggested mechanism of inhibition is due to the formation of a complex with the nicotinamide ring of NAD. Such a mechanism cannot account for the inhibition of poly(ADP-ribose) synthetase by 3-aminobenzoic acid because 2-aminobenzoic acid and 4-aminobenzoic acid were equally potent inhibitors of NAD glycohydrolase but had no effect on poly(ADP-ribose) synthetase activity (Table 8).

A second possible explanation is that the amino group may be acting as a nucleophile and hydrolysing the ester linkage between ADP-ribose and protein. This would decrease the acid-insoluble radioactivity and thus give an apparent inhibition. This was investigated by analysing the effect of 2mM 3-aminobenzamide acid on the acid-insoluble radioactivity in a reaction mixture after pre-labelling with ^{3}H-NAD and addition of urea to inhibit enzyme activity.

Figure 8 shows 3-aminobenzamide acid has no significant effect on the stability of acid-precipitable radioactivity even at a concentration of 2mM (40-fold greater than the concentration used in the assay). The lack of inhibition by the two isomers of 3-aminobenzoic acid would also discount this as a possible mechanism.

At present, the inhibition of poly(ADP-ribose) synthetase by 3-aminobenzoic acid cannot be explained. With this exception, however, the results presented show that benzoic acids do not inhibit the enzyme. The major differences between the amides and the acids are the lack of the amide nitrogen and the ionization of the acid groups. In view of the lower inhibition of poly(ADP-ribose) synthetase elicited by 3-nitrobenzamide and nicotinamide (3.3.1), the ionized carbonyl group may favour dissociation from the binding site.

3.3.4 The effect of other compounds on ADP-ribosylation

From the previous sections, the prime requirement for inhibition of poly(ADP-ribose) synthetase is a carbonyl group attached to a planar hydrophobic ring. Such compounds are known to act as radiosensitizers in anoxic cells (255,256). In view of the proposed role of ADP-ribosylation in the repair of DNA after treatment with DNA damaging agents and particularly the enhancement of cytotoxicity of such treatments by inhibitors of poly(ADP-ribose) synthetase, two radiosensitizers were tested for their ability to inhibit

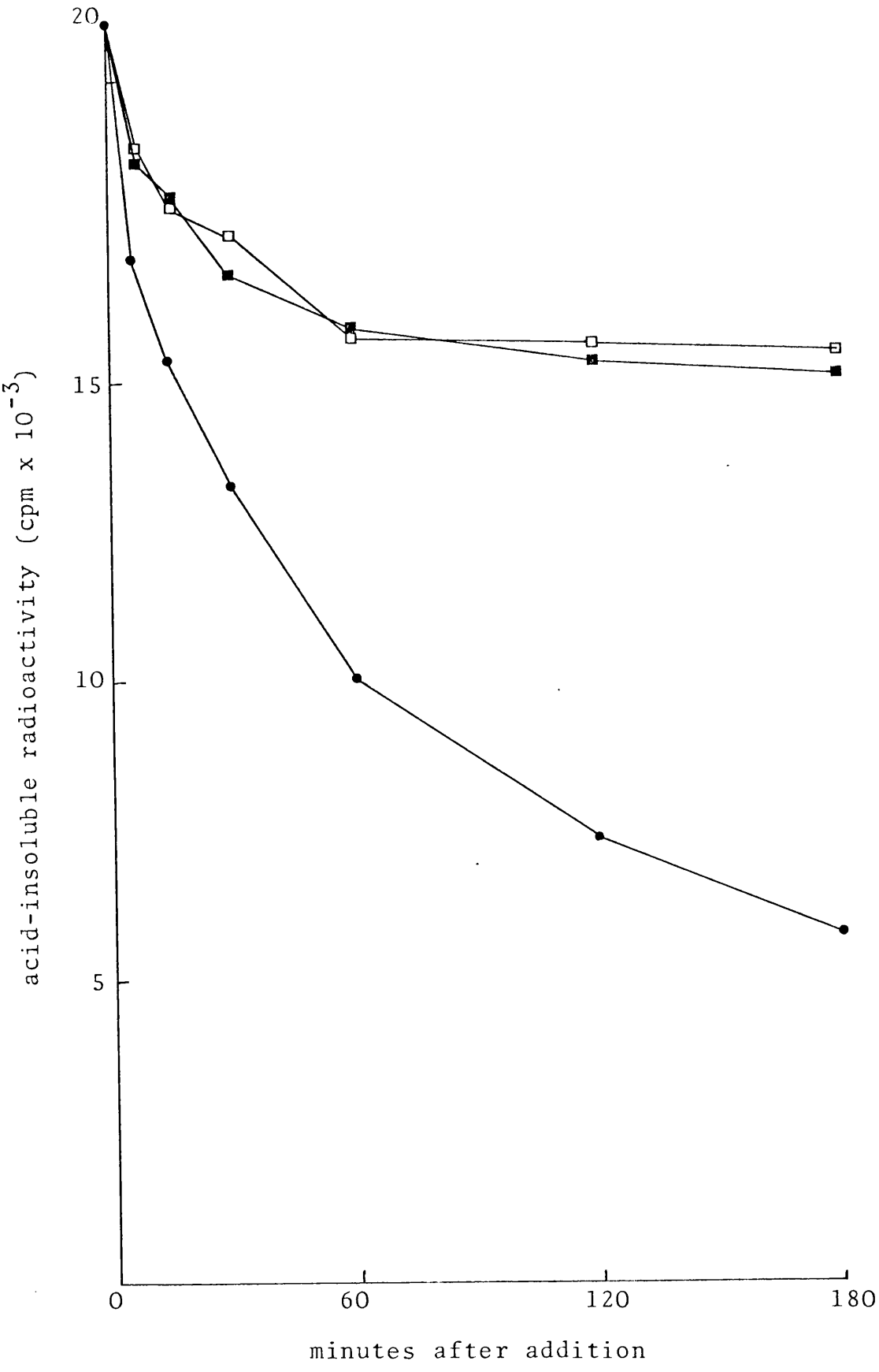


Figure 8 Effect of 3-aminobenzamide on stability of ADP-ribose - protein linkage

see Methods for experimental details

•, 2mM 3-aminobenzamide; □, 4M urea; ▪, both

poly(ADP-ribose) synthetase. As can be seen from Table 9, 4-nitroacetophenone had no effect on enzyme activity. Menadione (2-methylnaphthoqinone) inhibited the enzyme to a significant degree. Adams (255) has suggested that these compounds enhance the cytotoxicity of radiation by the electron affinic properties of the phenone group (ie C_6H_x-CO-). In the case of menadione, however, the enhancement of cytotoxicity may be mediated in part by inhibition of poly(ADP-ribose) synthetase.

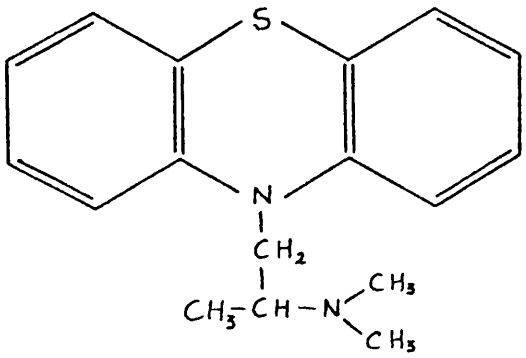
The mechanism of inhibition by menadione is unlikely to be the same as that produced by benzamides because of the rigid steric requirement. One possible explanation is the formation of a complex between menadione and the thiol group(s) of poly(ADP-ribose) synthetase. It has been shown that quinones are inhibitors of SH containing enzymes (257).

Abakumov & Lukienko (258) have shown that treatment of rats with either Promethazine, Hydrallazine or Obsidan (Fig. 9) caused an increase in the hepatic NAD concentration. The possibility that this increase is due to direct inhibition of poly(ADP-ribose) synthetase was investigated. Of the two drugs tested, only hydrallazine had a significant effect (Fig. 10).

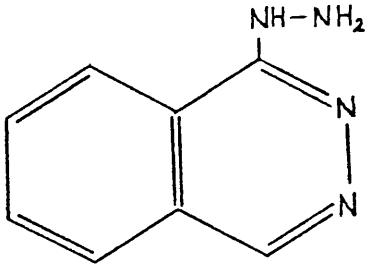
Table 9 Effect of radiosensitizers on poly(ADP-ribose) synthetase activity

The figures in parentheses are the percentage inhibition.

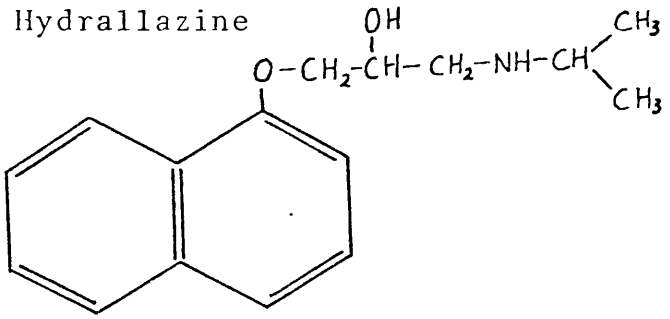
Addition		Incorporation of NAD into acid-insoluble material (nmol/min/mg protein)
Control		2.63
4-Nitroacetophenone	(50μM)	2.50 (5)
	(250μM)	2.62 (0)
Menadione	(50μM)	2.00 (24)
	(250μM)	0.53 (80)



Promethazine



Hydrallazine



Obsidan

Figure 9 The structure of promethazine, hydrallazine and obsidan.

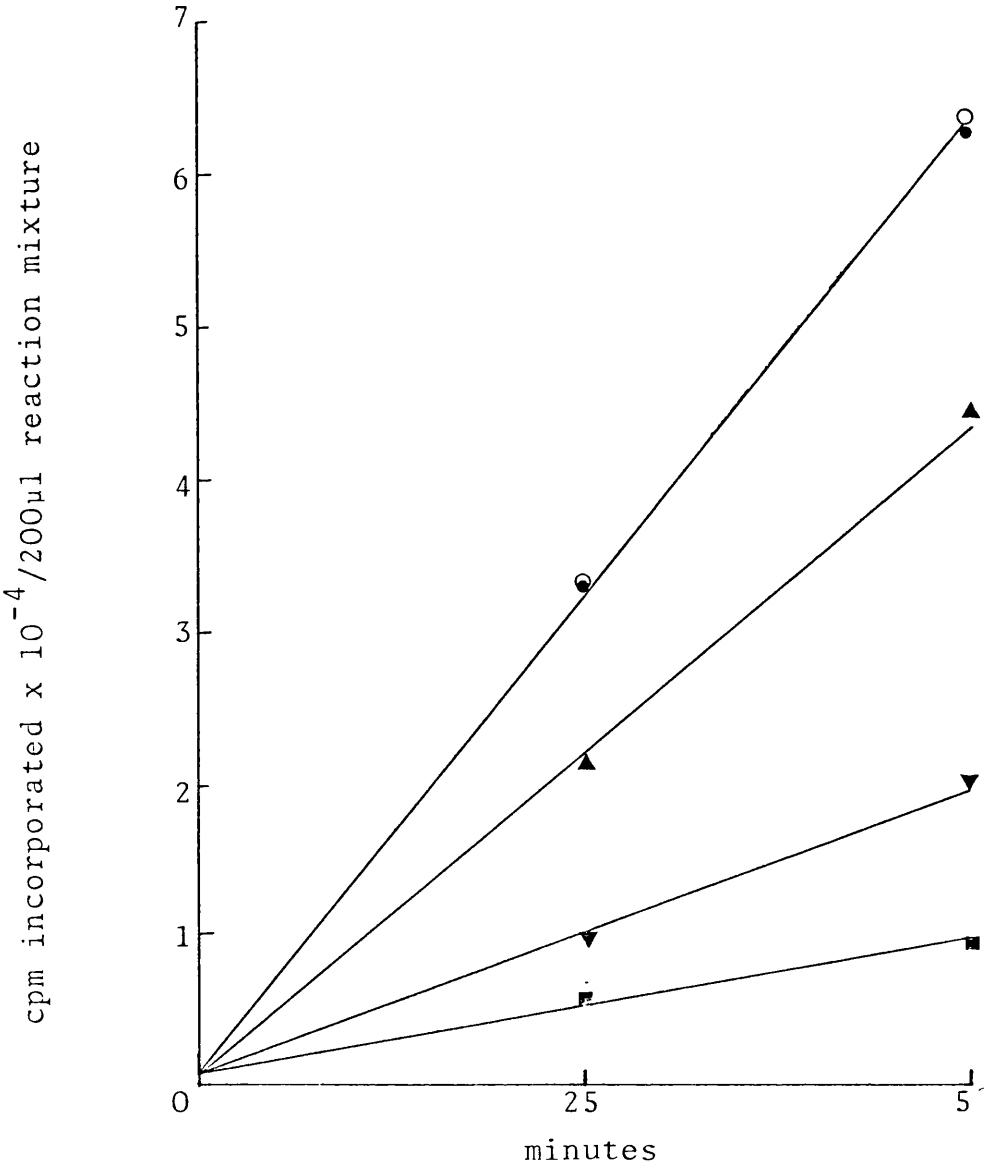


Figure 10 Effect of hydrallazine and promethazine on the incorporation of ^{3}H-NAD into acid-insoluble material.

Assays were performed as described in Methods section.

- ● control
- ○ 100µM promethazine
- ▲ 25µM hydrallazine
- ▼ 50µM "
- ■ 100µM "

SECTION 4

THE EFFECT OF 3-AMINOBENZAMIDE ON L1210 CELLS

4.1. Introduction

3-Aminobenzamide was chosen as a probe for the biological function of ADP-ribosylation for several reasons. It is the least hydrophobic of the benzamides tested. It also has the advantage that it exhibits fluorescence when viewed under u.v. light. This allows semi-quantitative studies on the level and metabolism of 3-aminobenzamide in the cell.

Initial studies with 3-aminobenzamide were performed on the slime mould, Physarum polycephalum, and the human cell line, Burkitt's lymphoma (Daudi). These latter were abandoned as model systems because of problems with availability. The following section describes the effect of 3-aminobenzamide on L1210 cells, a murine lymphocytic leukaemic cell line (259). In view of the structural similarity between 3-aminobenzamide and nicotinamide, the effect of the former on enzymes involved in NAD metabolism was investigated. The stability in L1210 cells was studied, together with the effect of 3-aminobenzamide on parameters such as the rate of cell proliferation, the incorporation of radioactive precursors into macromolecules and the level of NAD in the cell.

4.2. Methods

Cell culture

L1210 cells were obtained from Mrs D. Ellis (University of Sussex) or from Flow Laboratories (Glasgow, U.K.). 50ml stock cultures were maintained in RPMI 1640 medium supplemented with 10% horse serum, 2mM glutamine and 100 1U/ml of both penicillin and streptomycin at 37°C under 5% CO_2 in air.

Stock cultures of 50ml were subcultured every other day by addition of fresh medium to give a density of 1 x 10^5 cells/ml. Under these conditions, the mean generation time was 12-14 hours. The cells entered stationary phase at 1.2-1.5 x 10^6 cells/ml.

Cell number was determined by phase contrast microscopy using an improved Neubauer haemocytometer and at least six separate determinations were performed on each sample; the mean value was then taken.

4.2.1 Poly(ADP-ribose) synthetase assay

Assay 1 (Isolated nuclei)

Nuclei were isolated by the method of Takakusu et al (260) as follows. 200ml of cells at approximately 5 x 10^5 cells/ml were harvested by centrifugation at 1,500 rpm for 10 minutes in an MSE bench centrifuge and washed twice with 10ml 0.15M sucrose; 5mM $CaCl_2$; 25mM Tris. HCl pH 7.5. The final pellet was resuspended in 5ml of the above solution. An equal volume of the above solution containing 0.25% sodium

deoxycholate and 0.5% Nonidet P40 was then added slowly and the suspension was gently shaken for 5 minutes. To this was added 50ml 0.15M sucrose; 5mM $CaCl_2$; 25mM Tris. HCl pH 7.5 and the nuclei harvested by centrifugation at 1,500 rpm for 15 minutes. The resulting nuclei were washed once in 10ml 0.25M sucrose; 5mM $CaCl_2$; 25mM Tris. HCl pH 7.5. They were finally resuspended at 1 x 10^8 nuclei/ml in 100mM Tris. HCl pH 8.3; 60mM KCl; 10mM $CaCl_2$; 4mM NaF; 2mM DTT.

Poly(ADP-ribose) synthetase activity was assayed as follows. 480µl 100mM Tris. HCl pH 8.3; 60mM KCl; 10mM $MgCl_2$; 4mM NaF; 2mM DTT; 10-250µM ^{3}H-NAD (sp. act. 200µCi/µmole) was equilibrated at 26°C for 5 minutes. The reaction was started by the addition of 2 x 10^6 nuclei (20µl). After 5 minutes, 2ml 20% (w/v) TCA was added and the mixture left on ice for at least 30 minutes. The acid-insoluble material was collected on Whatman GF/C filters, washed with 5 x 5ml 1% TCA and once with 10ml absolute ethanol. The filters were fried at 100°C and the radioactivity determined by liquid scintillation counting in 0.5% (w/v) PPO in toluene.

Assay 2 (Permeabilized cells)

L1210 cells were permeabilized by the method of Berger et al (124,125) except that sucrose was omitted from the permeabilization medium as recommended for L1210 cells (261).

Cells at 5 x 10^5/ml were harvested by centrifugation for 10 minutes at 1,500 rpm at 4°C. The cells were

resuspended at 2 x 10^6 cells/ml in permeabilization buffer (10mM Tris. HCl pH 7.8; 1mM EDTA; 30mM 2-mercaptoethanol; 4mM $MgCl_2$) and left on ice for 15 minutes. The cells were harvested as before and resuspended in permeabilization buffer at 2 x 10^7 cells/ml. Over 90% of the cells were rendered permeable to trypan blue by this method.

Poly(ADP-ribose) synthetase was assayed by the addition of 2 x 10^6 cells (100µl) to 50µl 120mM $MgCl_2$; 100mM Tris. HCl pH 7.8; 1mM or 3mM ^{3}H-NAD (33µCi/µmole) which had been pre-incubated at 30°C. At various times 40µl aliquots were removed and applied to filter discs presoaked in 20% TCA in ether and dried. The processing was identical to that described in Section 3.2.2.

4.2.2 NAD glycohydrolase

Cells at a density of 5 x 10^5 cells/ml were harvested as described previously. They were washed with 10ml 0.15M NaCl; 5mM EDTA; 10mM Tris. HCl pH 7.4 twice. The pellet was finally resuspended in 5mM EDTA; 10mM Tris. HCl pH 7.4 at 2 x 10^7 cells/ml and left on ice for 10 minutes. They were then briefly sonicated (10 seconds in a "Sonicleaner" sonicating water bath). The sonicate was used for the assay of NAD glycohydrolase.

The assay consisted on the addition of 100µl of the sonicate (the equivalent of 2 x 10^6 cells) to 50µl 0.15M NaCl; 5mM EDTA; Tris. HCl pH 7.4; 1.5mM (carbonyl-^{14}C) NAD

s.a.1.3μCi/μmole at 37°C. At various times, 30μl of the reaction mixture was added to 30μl ethanol and left on ice.

10μl of the suspension was applied to a PEI-cellulose t.l.c. plate in a 1cm strip.1.5cm above the bottom edge. Nicotinamide was added as a marker. The t.l.c. plate was developed in butanol; methanol; water; conc. NH_4OH (60:20:20:1) until the solvent front was 5cm above the origin. The plate was dried and the position of nicotinamide was marked (Rf 0.66). A 1cm strip which included the nicotinamide spot was cut out together with a 1cm strip from the origin which contained the NAD spots. The radioactivity in each was determined by scintillation counting in 4ml 0.5% PPO in toluene. The amount of nicotinamide produced was calculated from the percentage of radioactivity in the nicotinamide spot relative to the total radioactivity in both spots after background cpm had been subtracted.

For characterization of the reaction products, an aliquot of the above was applied to a PEI-cellulose t.l.c. plate which was developed in ethanol: 1M NH_4Ac pH 5 (70:30). After drying, the plate was cut into 0.5cm strips and the radioactivity determined as above.

For the identification of ADP-ribose as a reaction product the assay was performed as above except that [^{3}H-ade]-NAD was used as the radiolabel. Aliquots were analysed by t.l.c. on PEI-cellulose. The plate was developed in 1M acetic acid until the solvent front was 2cm above the origin. It was then developed in 0.9M acetic acid;

0.3M LiCl for a further 10cm. The plate was dried and cut into 0.5cm strips. The strips were placed in scintillation vials containing 1ml 5% (v/v) perchloric acid and heated at 75°C for 15 minutes. The vials were allowed to cool, 10ml Triton X100; toluene; PPO (30:70:0.5; v/v/w) was added and the radioactivity determined.

4.2.3 Nicotinamide mononucleotide pyrophosphorylase assay

150ml of cells at 0.5 x 10^6/ml were harvested as before and washed twice with 0.15M NaCl; 10mM Tris. HCl pH 7.4. The pellet was resuspended in 0.5ml 50mM Tris. HCl pH 8.3 and the cells broken by freezing and thawing twice. The lysate was centrifuged at 20,000 rpm for 60 minutes in a Beckman SW50.1 rotor. The supernatant was used for the assay below.

20µl of the supernatant was added to 10µl 1mM ^{14}C-nicotinamide (sp. act. 50µCi/µmol) and 20µl 50mM Tris. HCl pH 8.3; 100mM $MgCl_2$; 2.1mM phosphoribosylpyrophosphate; 3.25mM ATP; 2.9mM AMP. After various times at 37°C, 50µl absolute ethanol was added and the mixture was put on ice. Aliquots were analysed as for the NAD glycohydrolase assay.

4.2.4 NAD pyrophosphorylase assay

200ml of cells at 0.5 x 10^6/ml were harvested and nuclei were isolated as described in Section 4.2.1 except that the final pellet was resuspended in 100mM Tris. HCl

pH 7.8 at 1 x 10^8 nuclei/ml.

100µl of the above was added to 100µl 100mM Tris. HCl pH 7.8; 20mM $MgCl_2$; 10mM ATP; 4mM NMN; 20mM AMP; 20mM nicotinamide. After various times at 37°C, 20µl of the reaction mixture was added to 180µl 55% (v/v) ethanol and left on ice until assayed for NAD by the method of Nisselbaum & Green (262) as described below.

1.95ml 100mM glycyl glycine buffer pH 7.4; 100mM nicotinamide; 0.5M ethanol, 800µl (1mg/ml) phenazine methyl sulphate, 50µl (5mg/ml) thiazoyl blue and 100µl 1mg/ml yeast alcohol dehydrogenase (400 1U/mg) were pre-incubated at 37°C for 5 minutes in subdued light. 50µl of the sample was added and the change in absorbance at 570nm was monitored for 5 minutes using a Pye-Unicam SP 8-100 dual beam spectrophotometer. The change in OD was compared with the rates obtained using known amounts of NAD.

4.2.5 Entry of 3-aminobenzamide into L1210 cells and the metabolism of 3-aminobenzamide

A solution of 100mM 3-aminobenzamide in 150mM NaCl was sterilized by autoclaving at 15 p.s.i. for 15 minutes.

3-Aminobenzamide was added to 200ml cultures in exponential growth phase to a final concentration of 2mM. The cell density was approximately 5 x 10^5 cells/ml. At various times after the addition of 3-aminobenzamide, 30ml aliquots were taken, centrifuged at 2,000 rpm for

1 minute. The pellets were washed once with 20ml 0.15M NaCl. 0.5ml methanol was added to the pellet which was dispersed by sonication. Aliquots (10 or 20µl) of this suspension were applied as 1cm strips to a celluloset t.l.c. plate which was developed with butanol; methanol; water; NH_4OH (60:20:20:1; by vol.) until the solvent front had reached 5-6cm above the origin. After drying, the intensity of the blue fluorescent spots was compared with a series of known amounts of 3-aminobenzamide which had been chromatographed under similar conditions. The sensitivity of the method was approximately 0.05-0.1 nmoles (0.068µg).

4.2.6 Effect of 3-aminobenzamide on cell proliferation

Cells were subcultured to a density of 0.5 x 10^5 cells/ml. After 16-18 hours, 2ml sterile 3-aminobenzamide (10mM-200mM in 0.15M NaCl) or 0.15M NaCl was added to 18ml of cells. At various times after the addition of 3-aminobenzamide, the cell number was determined.

4.2.7 Effect of 3-aminobenzamide on the incorporation of precursors into macromolecules

3-Aminobenzamide solution was added to give a final concentration of 2mM to exponentially dividing cultures at 2 x 10^5 cells/ml. 0.15M NaCl was added to control cultures. At various times after the addition of 3-aminobenzamide,

0.5ml aliquots were removed and added to 0.5μCi of ^{3}H-thymidine, ^{3}H-adenosine or ^{3}H-leucine, and then incubated at 37°C. 50μl aliquots were removed at 20, 40 and 60 minutes after the addition of isotope and applied to acid soaked discs in the case of thymidine, adenosine and uridine labelling. The discs were then processed as described in Section 3.2. The aliquots from the incorporation of ^{3}H-leucine were added to 50μl 10% TCA and heated at 70°C for 15 minutes. After cooling on ice, 50μl was applied to paper discs and processed as above.

4.2.8 NAD levels in L1210 cells

Cultures were grown for various times in the presence or absence of 2mM 3-aminobenzamide. Aliquots (30ml) were harvested and 2ml 0.5M $HClO_4$ was added. After 30 minutes on ice, they were centrifuged at 3,000 rpm for 15 minutes. The supernatents were then incubated at 37°C for 30 minutes, after which time 0.5ml 2M NaOH was added and the neutralized supernatents kept on ice until assayed for NAD as described in Section 4.2.4 except that standards contained an equivalent amount of 0.4M $NaClO_4$.

4.2.9 Poly(ADP-ribose) synthetase activity in permeabilized cells following treatment with 3-aminobenzamide in vivo

Cells were permeabilized and assayed for poly(ADP-ribose) synthetase activity as described previously except that cells were first washed with 0.15M NaCl; 10mM Tris. HCl pH 7.5.

4.3. Results and Discussion

4.3.1 Poly(ADP-ribose) synthetase

The data in Fig. 11 show that 3-aminobenzamide is a potent inhibitor of poly(ADP-ribose) synthetase in isolated nuclei from L1210 cells and is more inhibitory than nicotinamide. In order to obtain a more realistic estimate of the effect of 3-aminobenzamide *in vivo*, the inhibition of poly(ADP-ribose) synthetase activity in permeabilized cells was investigated. The method of Berger *et al* (261) was used for permeabilization. Over 95% of the cells were rendered permeable to trypan blue using this method. The assay used by Berger contained 0.33mM NAD. The NAD levels in L1210 cells have been estimated to be 1mM (195,263). The incorporation of radioactivity into acid-insoluble material was examined using 0.33mM and 1mM NAD (Fig. 12). Both substrate concentrations gave linear rates with time.

The effect of various concentrations of 3-aminobenzamide on the enzyme activity was examined. Figure 13 shows that ADP-ribosylation is inhibited over 90% by 250μM 3-aminobenzamide. Figure 14 shows that 3-hydroxybenzamide is an equally potent inhibitor.

4.3.2 NAD glycohydrolase

NAD degradation was assayed by the production of ^{14}C-nicotinamide from (carbony-^{14}C)-NAD. A crude sonicate was used for the assay for a variety of reasons. The

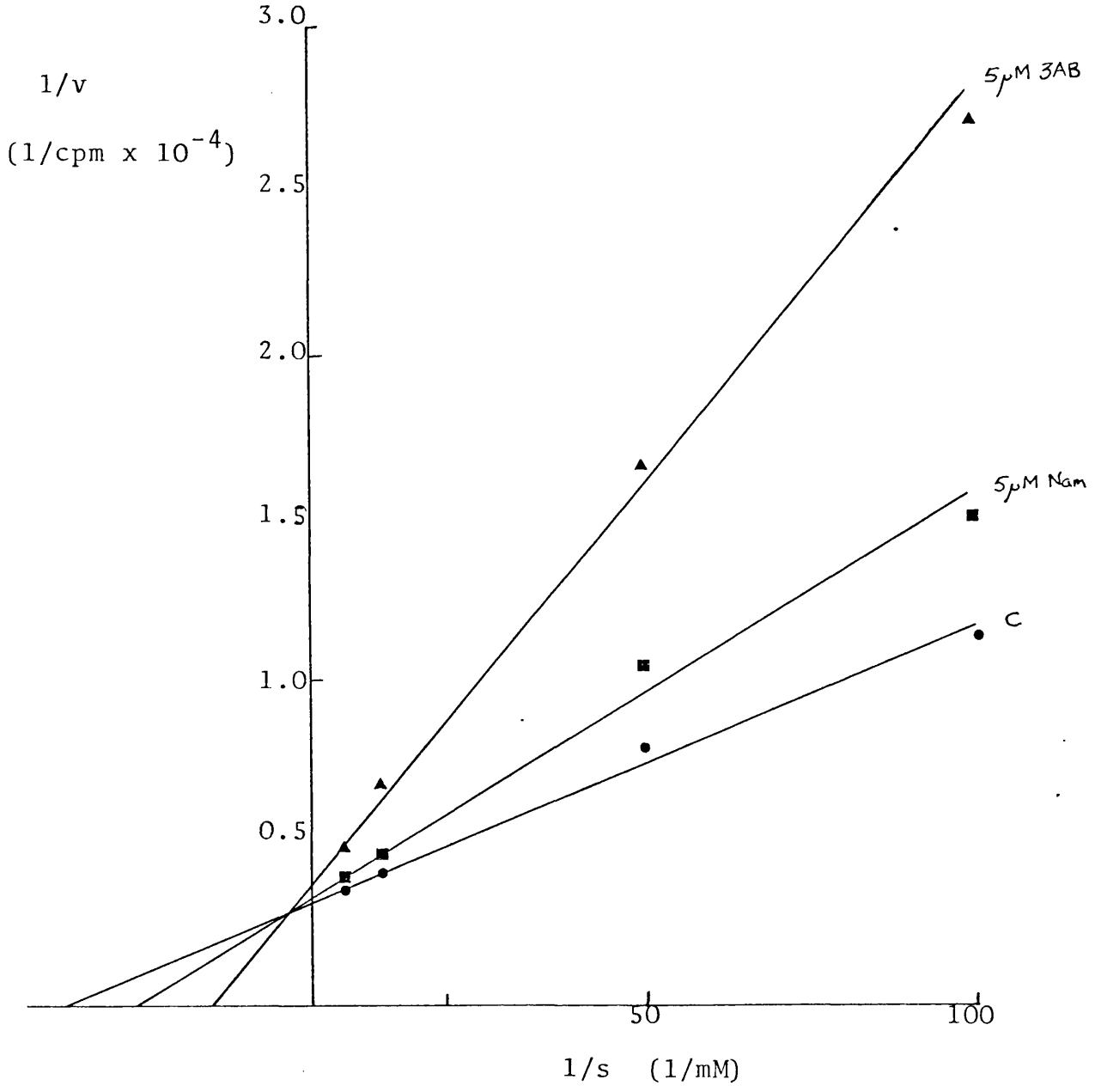


Figure 11 Lineweaver-Burke plot for ADP-ribosylation in L1210 isolated nuclei.

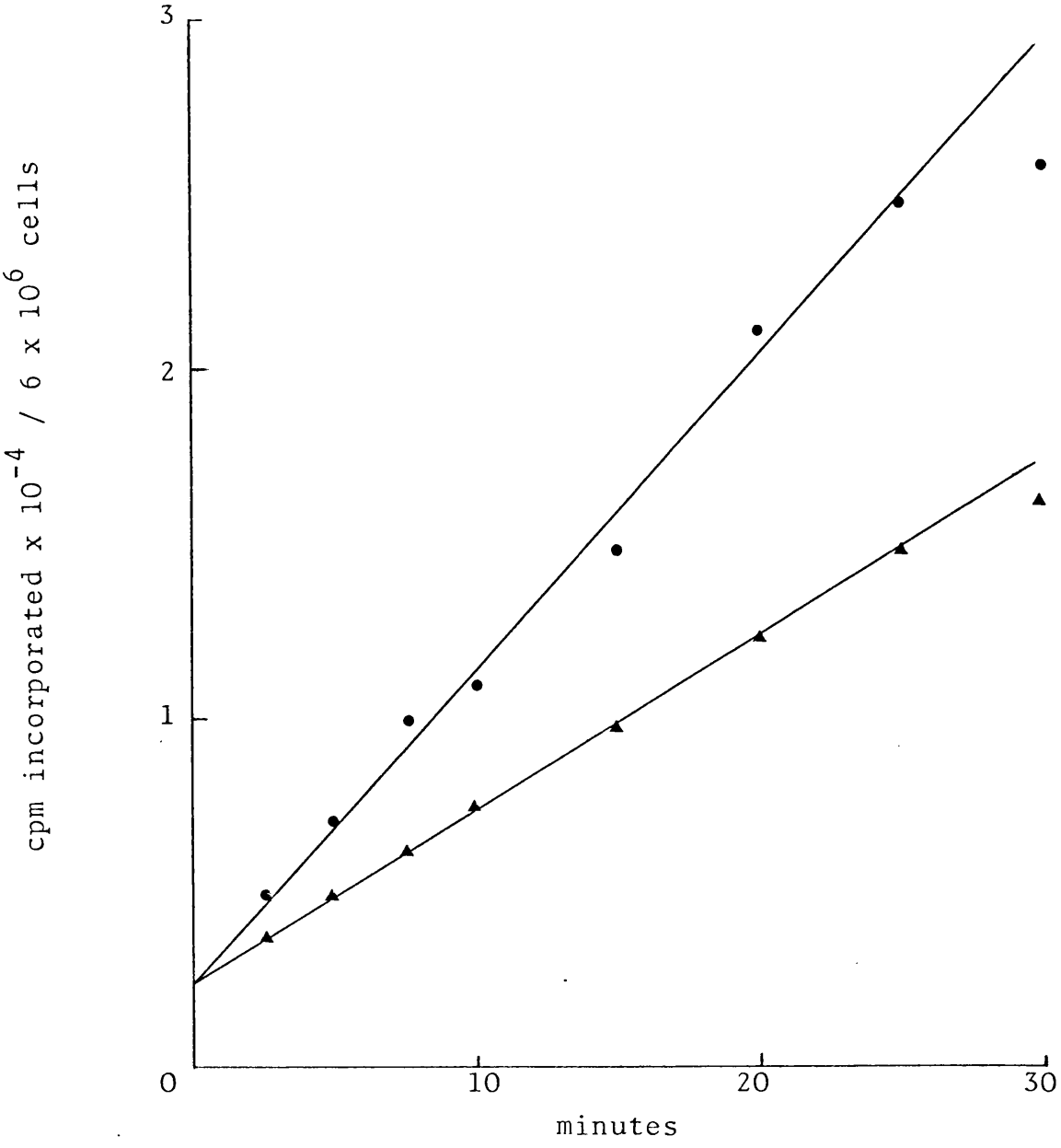


Figure 12 Time course of incorporation of ^{3}H-NAD into acid-insoluble material by permeabilised L1210 cells.

▲ 0.33mM NAD

• 1.0 mM NAD

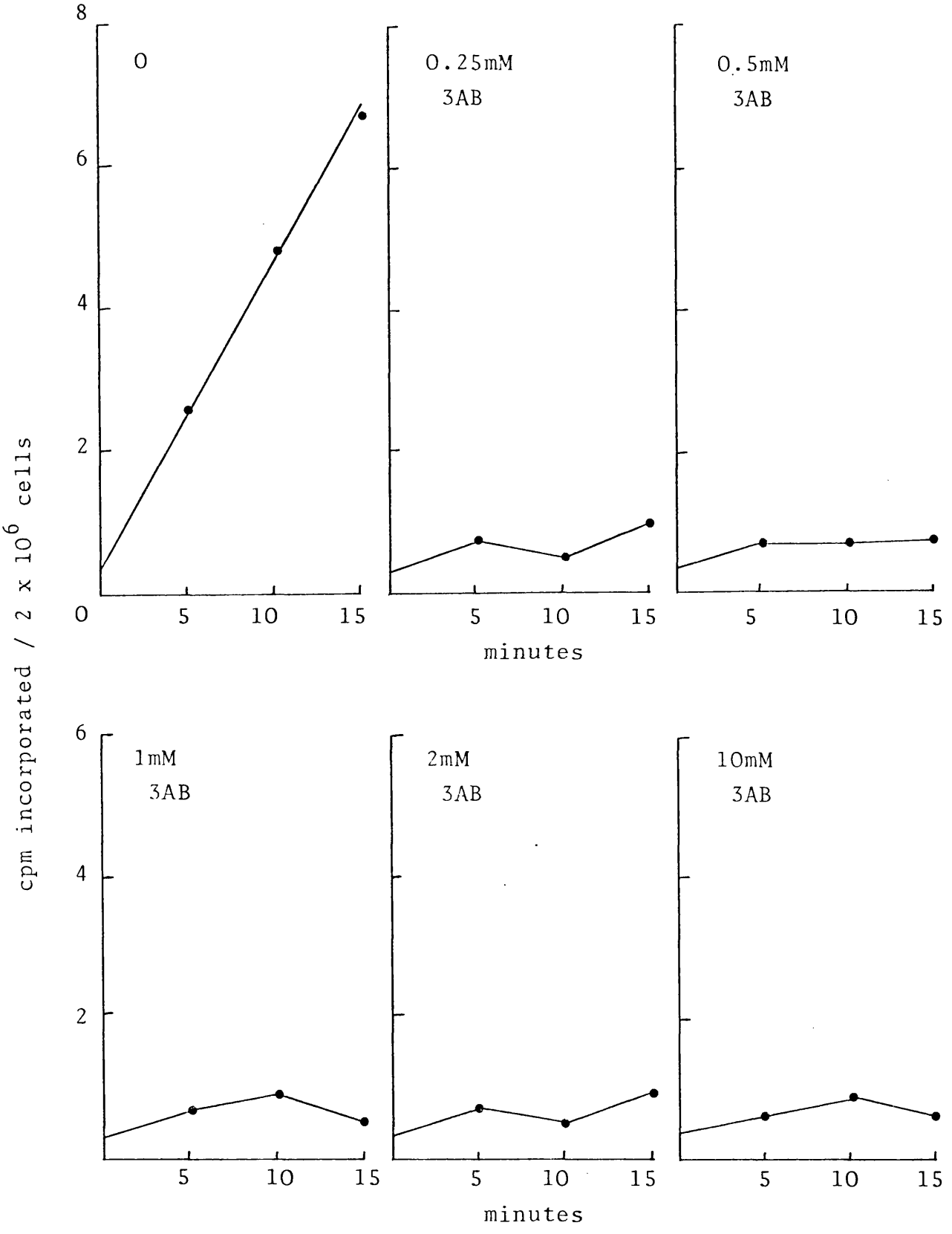


Figure 13 The effect of 3-aminobenzamide on ADP-ribosylation in permeabilised L1210 cells.

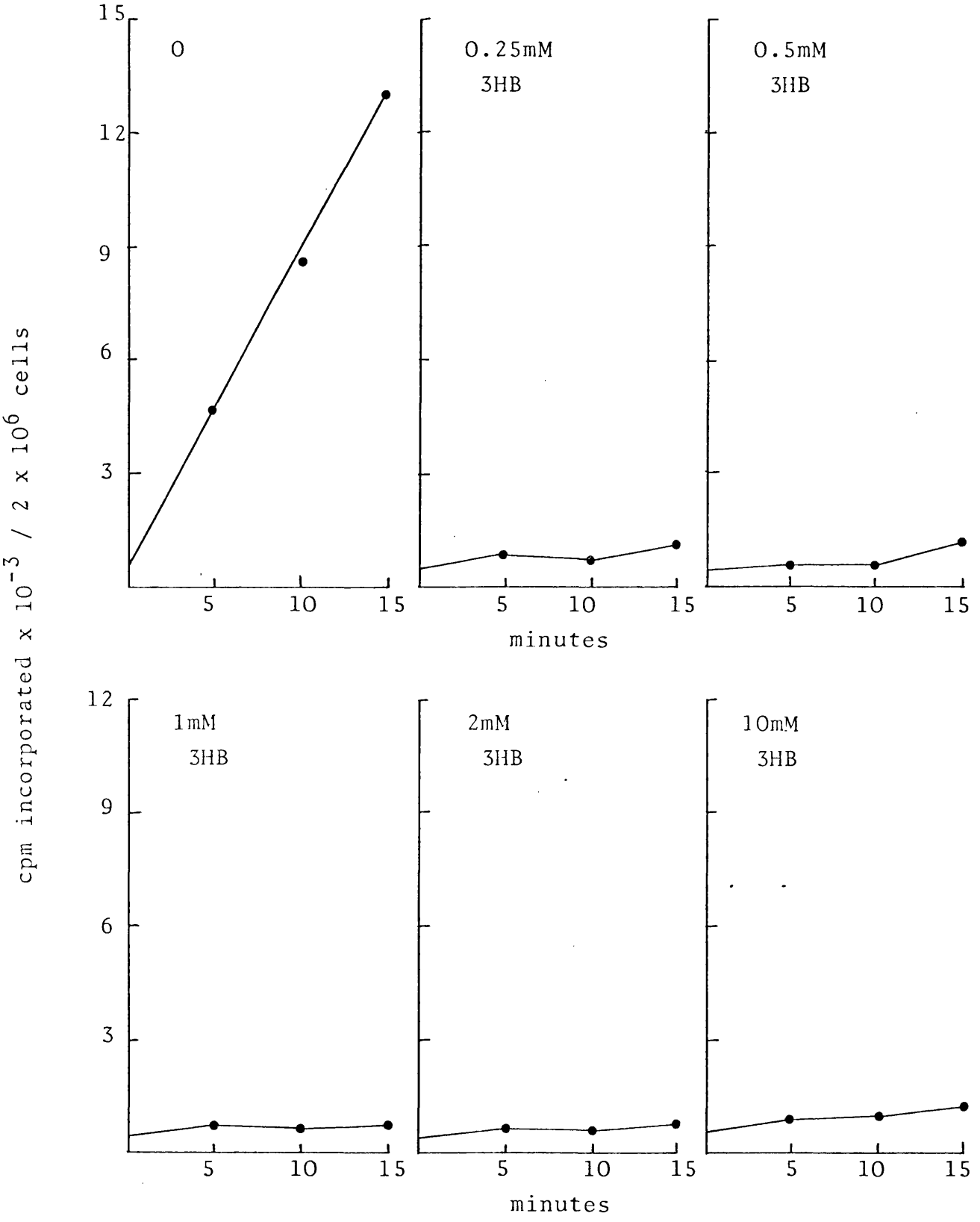


Figure 14 The effect of 3-hydroxybenzamide on ADP-ribosylation in permeabilised L1210 cells.

subcellular localization of NAD glycohydrolase has been described variously as microsomal (264), nuclear envelope (265,266), in secondary lysosomes (267) and in the plasma membrane (268-272). The location in the plasma membrane is noteworthy because it is thought to be an ectoenzyme, i.e. it is present on the outside surface of the cell. This has been established by comparison of the rate of NAD hydrolysis by homogenates and intact cells for E.A.T. cells (269) and bovine erythocytes (270). The enzyme from the microsomal fraction of calf spleen has been shown to be in the plasma membrane by density displacement of plasma membranes from other membranes by treatment with digitonin (268). The most convincing method has been the treatment of intact peripheral and spleenic macrophages with diazotized sulphanilic acid as an irreversible non-permeating enzyme inhibitor (272). Thus this form of the enzyme could have no role in the hydrolysis of intracellular NAD. Its function appears to involve the hydrolysis of extracellular NAD following endocytosis (273). The appearance of the enzyme in secondary lysosomes of rabbit liver following detergent treatment (267) is consistent with such a function.

One problem with the assay is the discrimination between NAD glycohydrolase and ADP-ribosyltransferases, which have been reported to be present in bovine and turkey erythrocytes (270,274-5),and poly(ADP-ribose) synthetase. This has been attempted by assaying permeabilized L1210 cells at low pH (236) and DNasel pretreatment to inactivate

poly(ADP-ribose) synthetase (269). One criticism of all these methods is that they may select one particular form of NAD glycohydrolase. Inhibition of poly(ADP-ribose) synthetase by mercurials or dithionitrobenzoic acid is unsuitable because NAD glycohydrolases from pig spleen (276) and rabbit liver lysosomes (267) are reported to possess essential thiol groups. 5mM EDTA was included in the assay medium to inhibit poly(ADP-ribose) synthetase which is stimulated by Mg^{2+}(1,248). To date no NAD glycohydrolases have been reported to require divalent cations; indeed the enzyme from pig spleen is inhibited by Mg^{2+} (276).

Figure 15 shows the time course of NAD hydrolysis in the presence and absence of 2mM 3-aminobenzamide. The inhibition by 3-aminobenzamide was investigated using [^{3}H-Ade]-NAD as substrate. Figures 16 and 17 show the reaction products after 5 minutes. The major difference is the size of the peak on the origin. This probably represents protein-bound material which is absent in the presence of 3-aminobenzamide. The most likely candidate is poly(ADP-ribose) synthetase. Although the enzyme is stimulated by Mg^{2+}, it has recently been reported that poly(ADP-ribose) synthetase from brain (277) is not inhibited by EDTA. From the data in Figure 15, nicotinamide production from NAD is inhibited 40% by the presence of 2mM 3-aminobenzamide after 5 minutes. A similar figure is obtained if the data in Figures 16 and 17 are expressed as percentage NAD hydrolysed (i.e. 32%). By expressing the same data as percentage ADP-ribose produced an inhibition of only 12% is

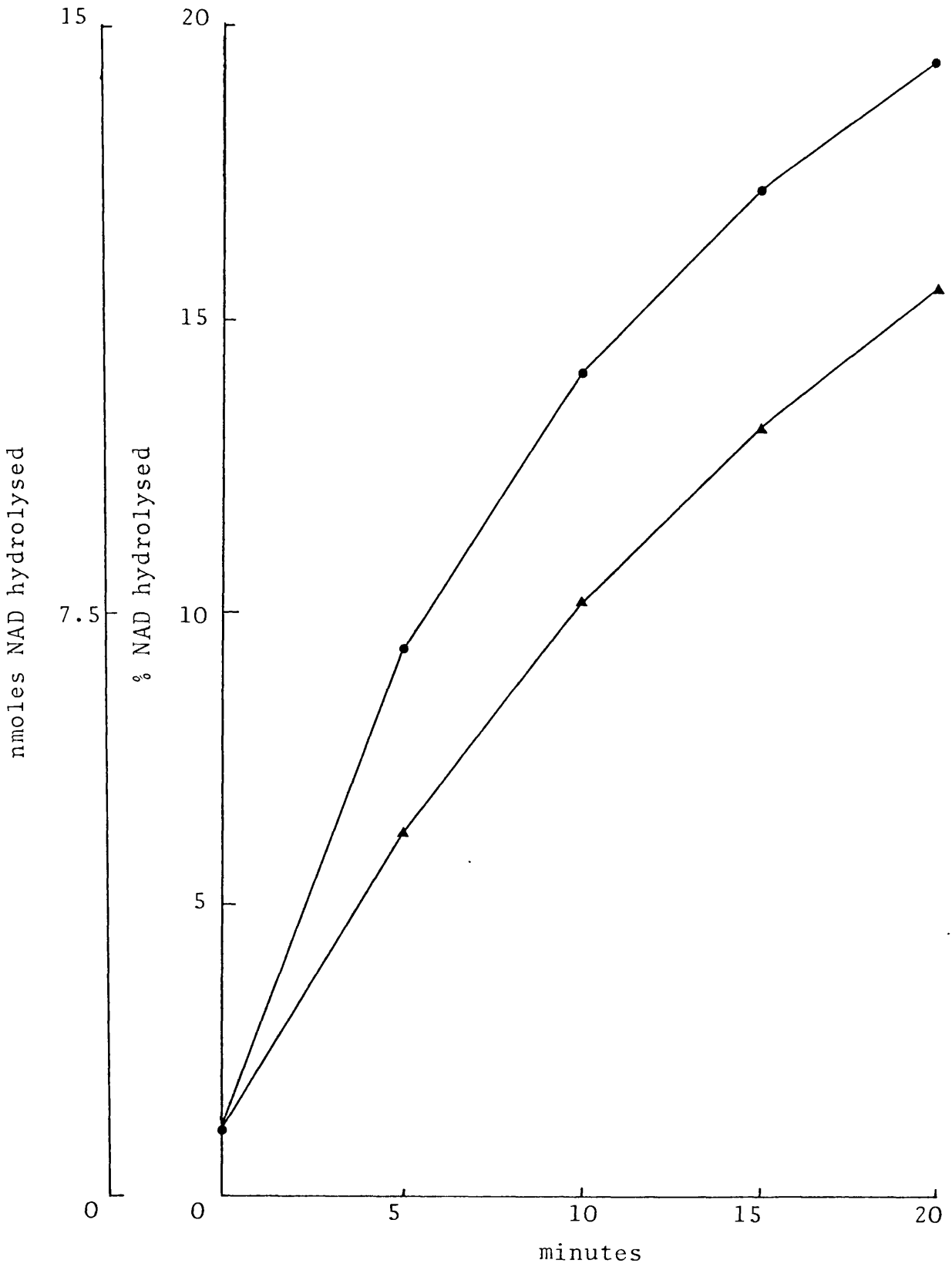


Figure 15 Effect of 2mM 3-aminobenzamide on NAD hydrolysis by sonicated L1210 cells.

• control; ▲ +2mM 3-aminobenzamide

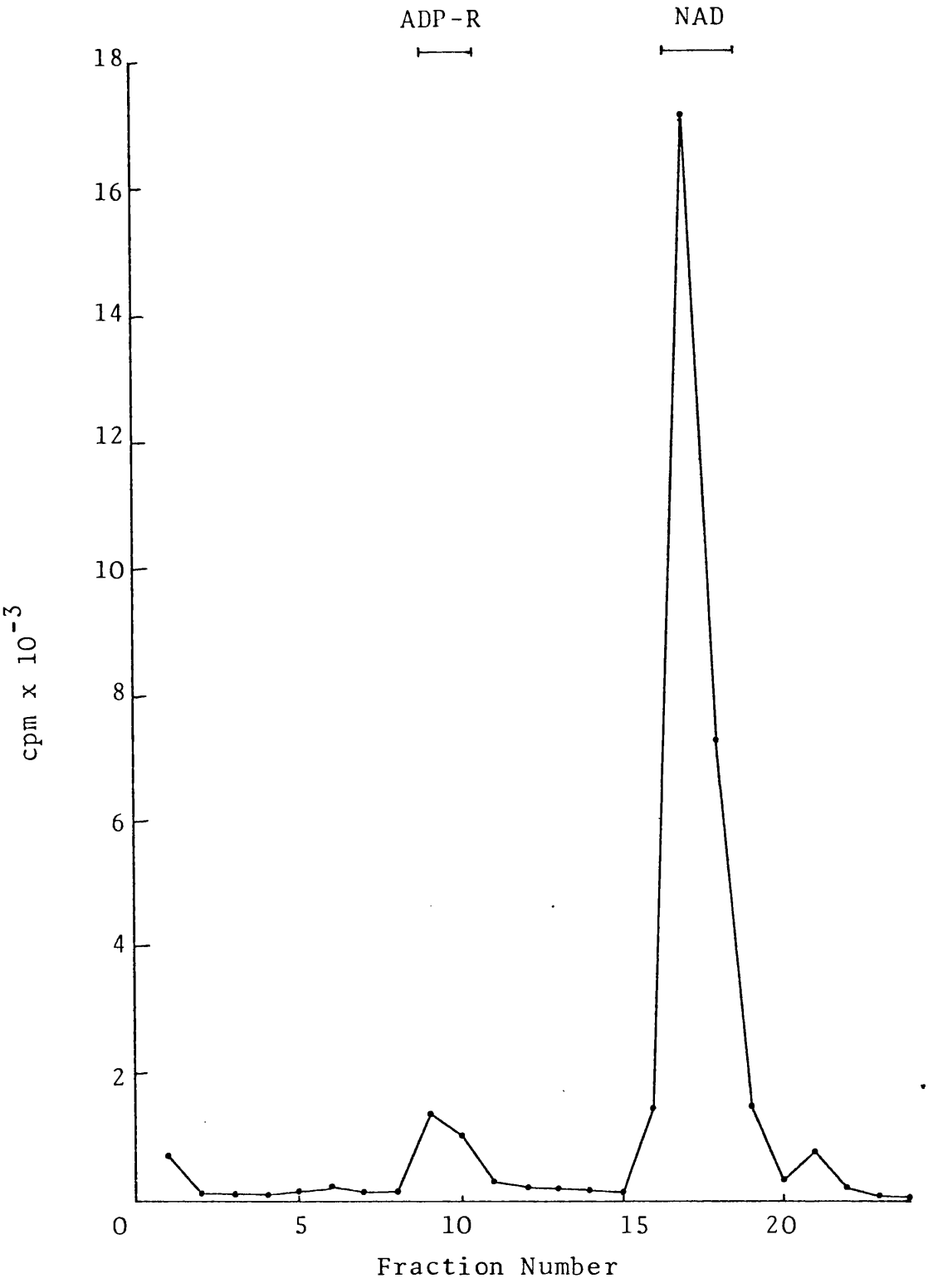


Figure 16 PEI-cellulose t.l.c. profile of reaction products after 5 minutes incubation.

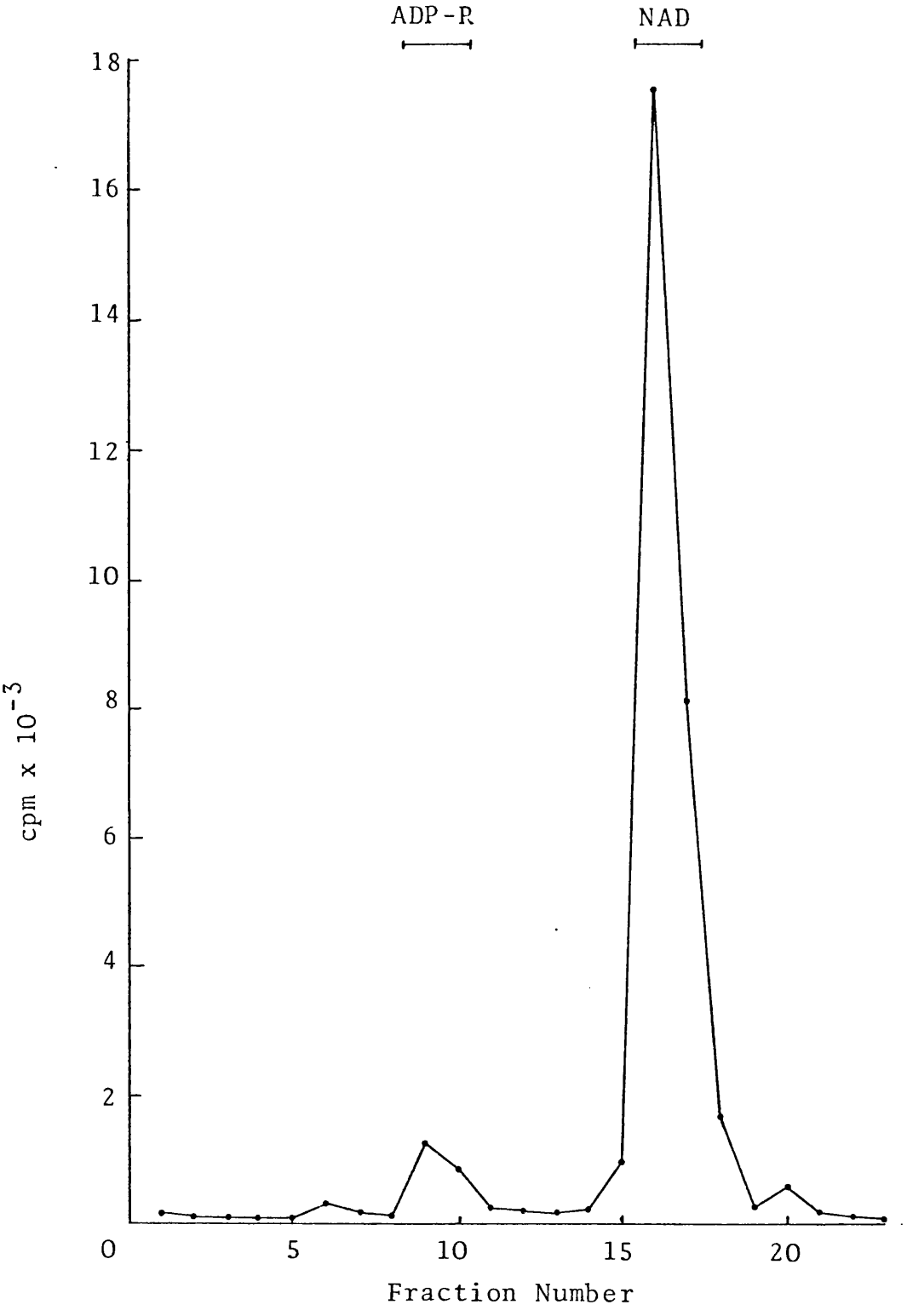


Figure 17 PEI-cellulose t.l.c. profile of reaction products after 5 minutes incubation in the presence of 2mM 3-aminobenzamide.

obtained. This probably represents an overestimate because no account has been taken of the hydrolysis of the linkage between ADP-ribose and protein. Thus it can be concluded that 2mM 3-aminobenzamide does not inhibit NAD glycohydrolase in these cells. Durkacz et al (195) have reported a similar finding. As early as 1945, it was shown that 160mM benzamide had no effect on NAD glycohydrolase from rabbit brain (277). Pyridine compounds including nicotinamide are known to inhibit NAD glycohydrolase (e.g. 270). Thus it appears that in contrast to poly(ADP-ribose) synthetase the pyridine ring nitrogen is necessary for binding to the enzyme active site.

4.3.3 Nicotinamide mononucleotide pyrophosphorylase

Figure 18 shows that 2mM 3-aminobenzamide has no effect on NMN pyrophosphorylase. A similar result was obtained if nicotinic acid was used in place of nicotinamide (Fig. 19). This compares with finding of Dietrich's group that analogues of nicotinamide are potent inhibitors of NMN pyrophosphorylase from rat liver (279). Thionicotinamide and 3-acetylpyridine were able to act as substrates (279,280). In addition, if 3-acetylpyridine, 6-aminonicotinamide or thionicotinamide were administered to rats, the corresponding NAD analogues were found in brain. Thus if these compounds are used as inhibitors of poly(ADP-ribose) synthetase in vivo, the effect cannot be ascribed directly

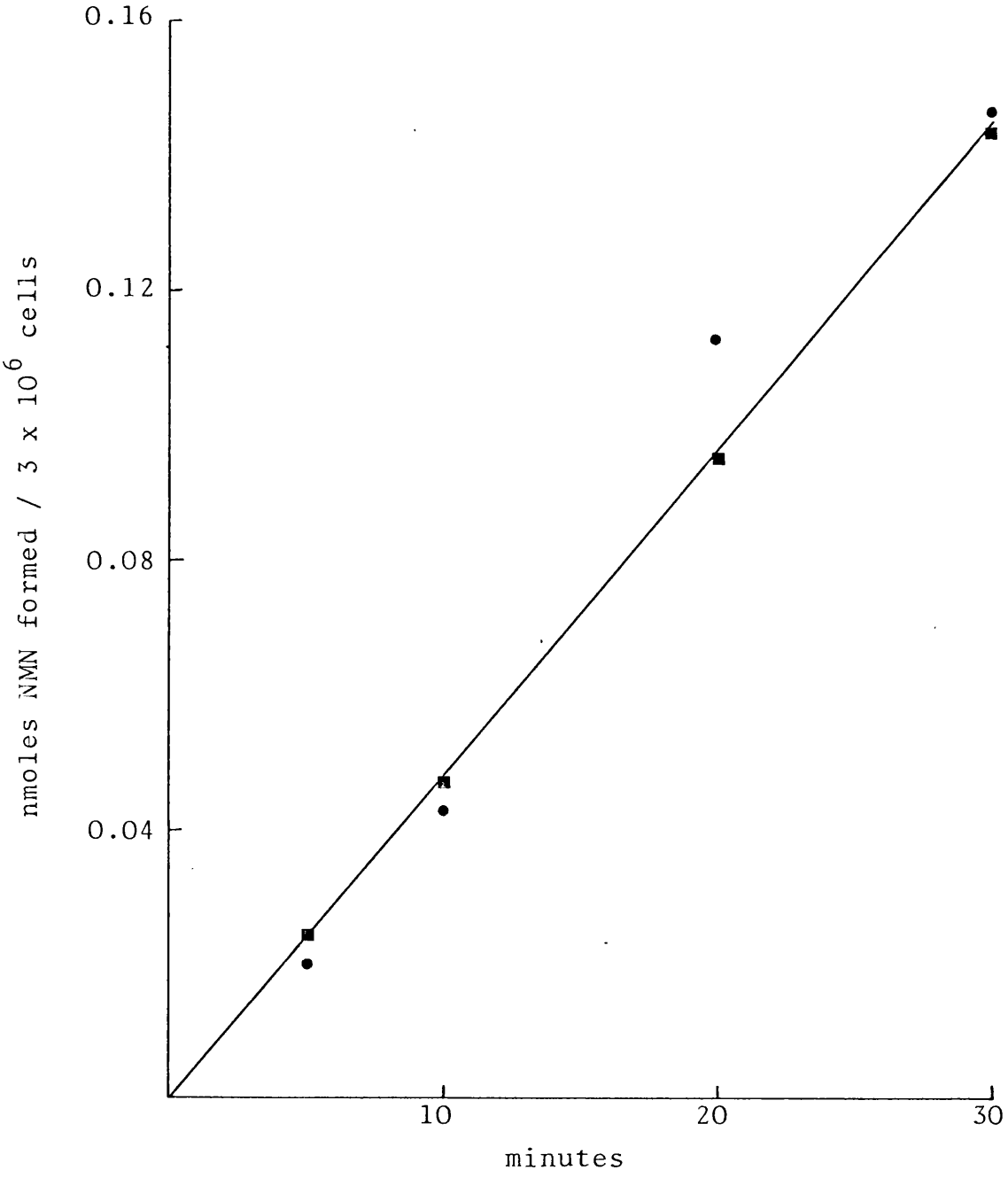


Figure 18 The effect of 2mM 3-aminobenzamide on NMN pyrophosphorylase activity.

■, control; ●, 2mM 3-aminobenzamide

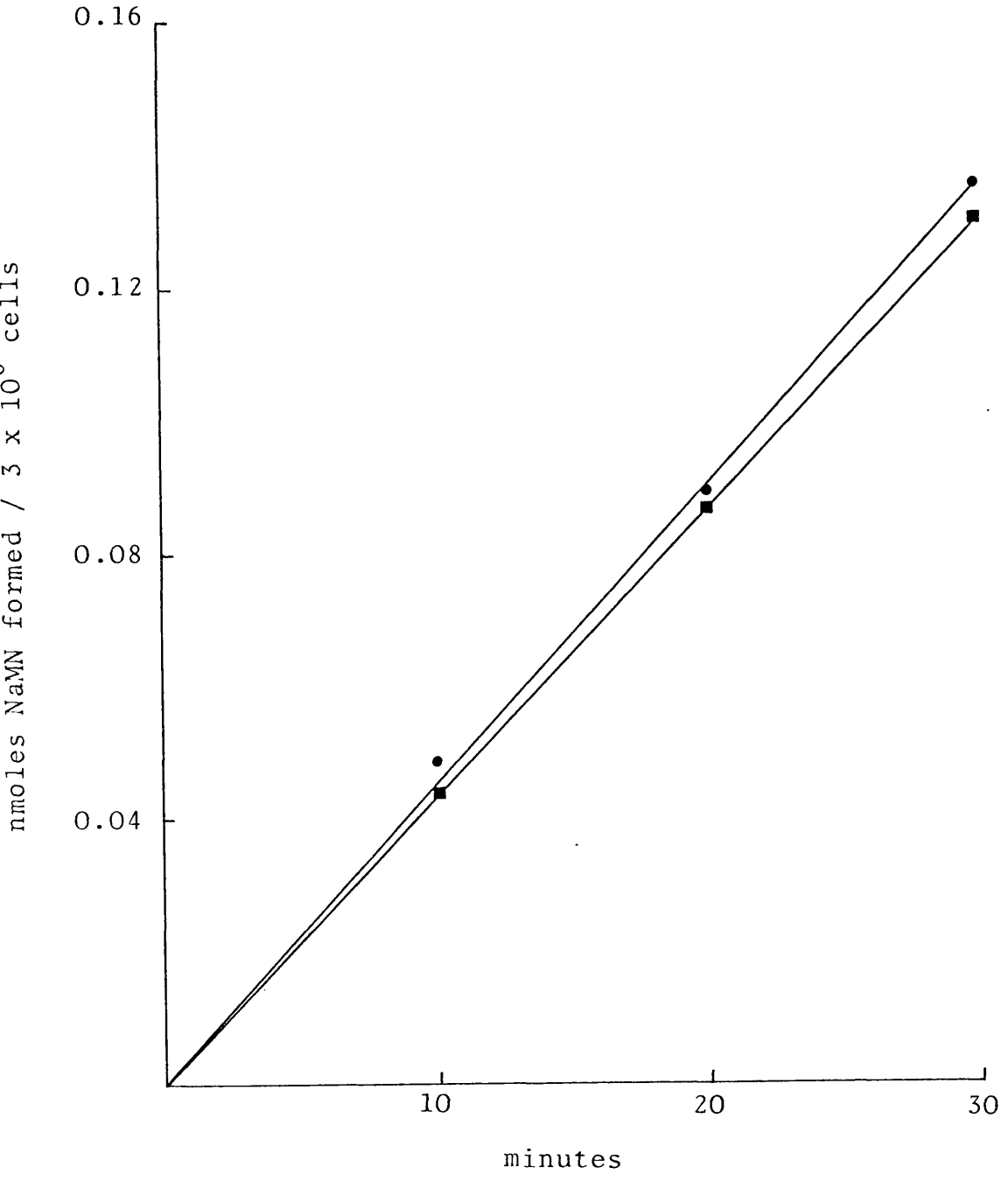


Figure 19 The effect of 2mM 3-aminobenzamide on NaMN pyrophosphorylase activity.

• control ■ 2mM 3-aminobenzamide

to inhibition of poly(ADP-ribose) synthetase.

The induction of enzymes in liver by nicotinamide and its analogues (281) is unlikely to be a direct effect on poly(ADP-ribose) synthetase since the majority of these compounds do not inhibit ADP-ribosylation (96).

4.3.4 NAD pyrophosphorylase

3-Aminobenzamide at a concentration of 2mM has no effect on NAD pyrophosphorylase (Fig. 20). This finding, together with the data presented earlier on the effect of 3-aminobenzamide on other enzymes involved in NAD metabolism in L1210 cells, means that any alterations in the level of NAD *in vivo* upon 3-aminobenzamide treatment are a consequence of the inhibition of poly(ADP-ribose) synthetase.

4.3.5 Entry of 3-aminobenzamide into the cell and metabolism

It must first be recognized that the technique used was extremely crude. It was however possible to show the appearance of a blue fluorescent spot after treatment of L1210 cells with 2mM 3-aminobenzamide *in vivo* when an aliquot of a methanolic cell extract was chromatographed on cellulose F t.l.c. plates. Even after a period of 48 hours had elapsed following the addition of 3-aminobenzamide to the cells, no other fluorescent spots could be detected.

If cells were treated with 2mM 3-aminobenzamide and an aliquot of the extract chromatographed and the intensity of

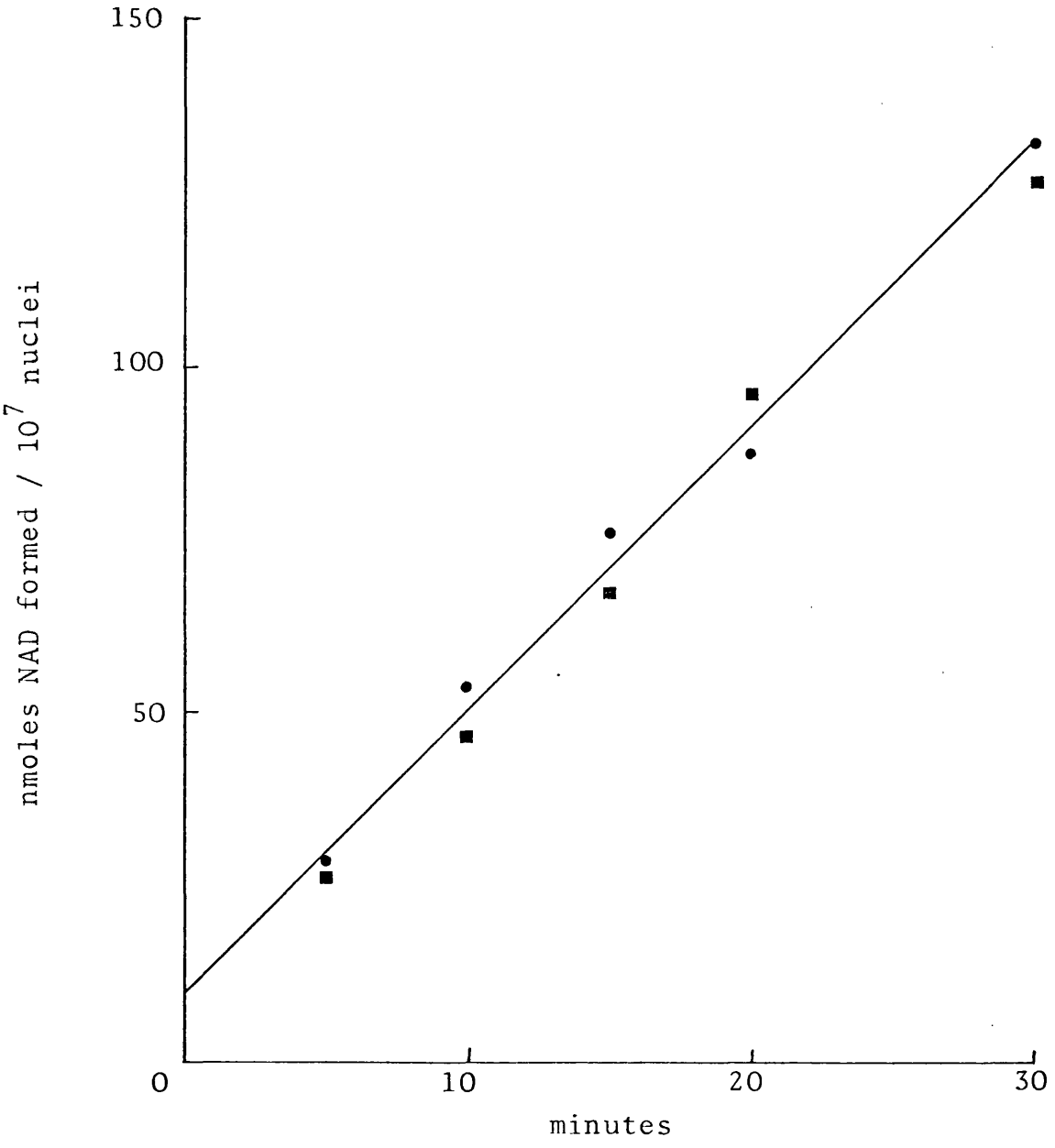


Figure 20 The effect of 2mM 3-aminobenzamide on NAD pyrophosphorylase activity.

•, control; ■, 2mM 3-aminobenzamide

the spot compared to know amounts of 3-aminobenzamide, the intracellular concentration was estimated to be approximately 0.8-1.2 nmoles/10^6 cells.

The lack of hydrolysis of 3-aminobenzamide is consistent with early work done by Thorpe and co-workers, who studied the metabolism of various benzamides in rabbits. From a number of studies they found the amide of 3-aminobenzamide was only hydrolysed 6-10% (282) compared to 80% for benzamide and 58% for 3-nitrobenzamide (283,284). 3-Aminobenzamide was also found to be relatively stable to hydrolysis in vitro by a rabbit liver extract (285). The main methods of metabolizing 3-aminobenzamide in rabbit were found to be acetylation of the amino group and hydroxylation of the benzene ring (282). If acetylation of 3-aminobenzamide occurs in L1210 cells, it would not be detected using the criterion of fluorescence because 3-acetylaminobenzamide lacks this property. For rigorous study of the metabolism of 3-aminobenzamide, it would be preferable to use radioactive material. The cost of obtaining [^{3}H]-3-aminobenzamide from the Radiochemical Centre, Amersham, U.K., made such an investigation impractical. For the purposes of studying ADP-ribosylation in L1210 cells, metabolism of 3-aminobenzamide by acetylation is of minor significance because 3-acetylaminobenzamide is a more potent inhibitor of poly(ADP-ribose) synthetase than the parent compound.

4.3.6 Effect of 3-aminobenzamide on cell proliferation

The effect of various concentrations of 3-aminobenzamide on the rate of cell division is shown in Figure 21. At concentrations of up to 5mM no significant difference in the cell number was observed. At concentrations above this 3-aminobenzamide caused a significant reduction in the rate of cell proliferation. This became more pronounced after 24 hours although after this time proliferation was almost completely inhibited. This could mean that 3-aminobenzamide inhibits proliferation after one cell division. Whether this effect is related to ADP-ribosylation of nuclear proteins is unsure since a concentration of 0.25mM inhibits ADP-ribosylation over 90% in permeabilized cells (Section 4.3.1).

Nduka *et al* (192) have investigated the cytotoxicity of various other inhibitors of poly(ADP-ribose) synthetase using L1210 cells. At 2mM, 5-methylnicotinamide and thymidine (in the presence of 3-mM deoxycytidine) had no effect on relative plating efficiency when L1210 cells were grown in soft agar. At concentrations above 2mM, 5-methylnicotinamide caused a rapid decrease in plating efficiency. All the methylxanthines were cytotoxic above 250μM, although the relative potency showed no correlation with their ability to inhibit poly(ADP-ribose) synthetase. Using the same technique, Durkacz *et al* (195) have recently reported that 3mM 3-aminobenzamide had no effect on plating efficiency.

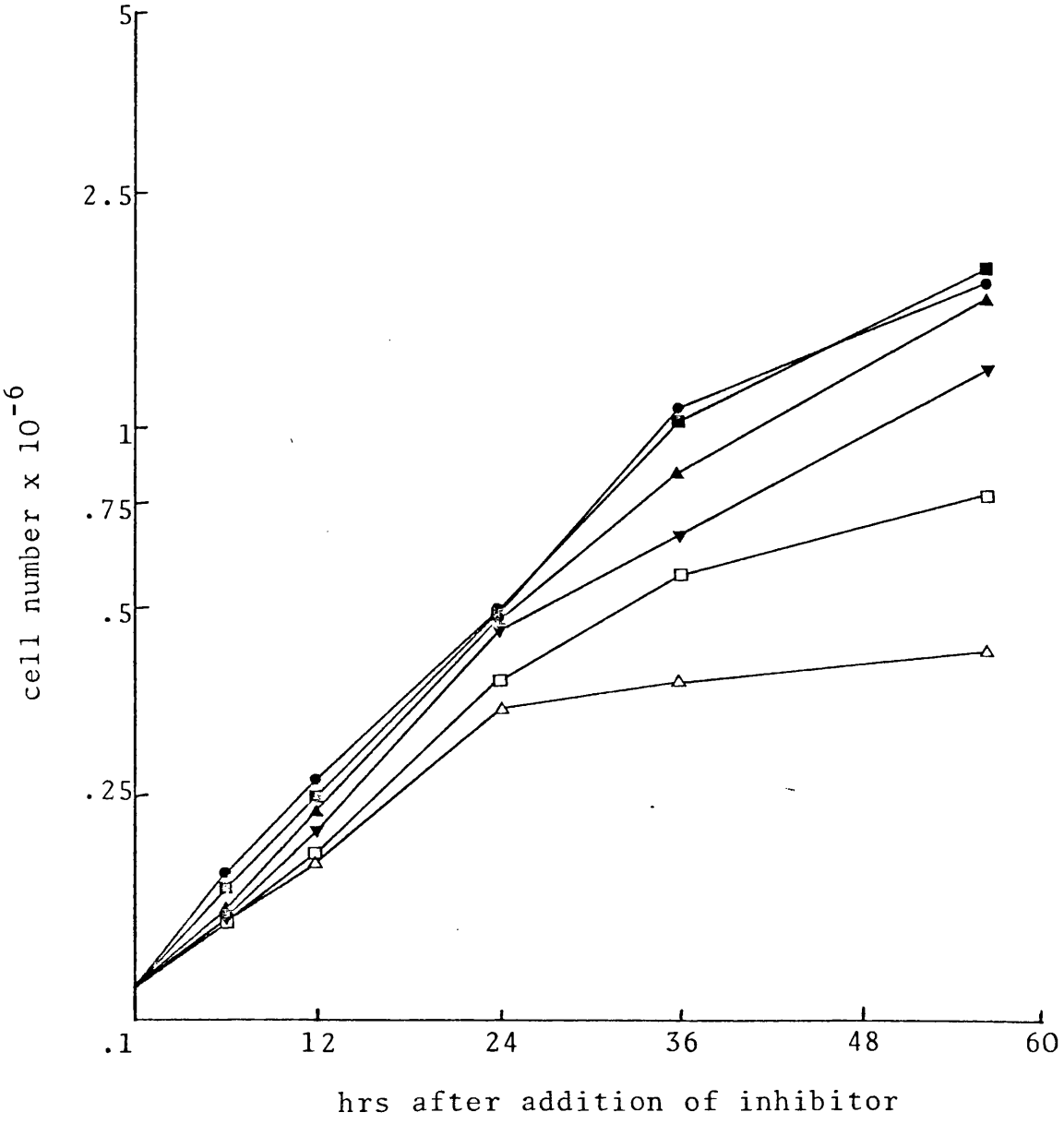


Figure 21 The effect of 3-aminobenzamide on proliferation of L1210 cells.

■ 0
● 2mM
▲ 5mM
▼ 10mM
□ 15mM
△ 20mM

Nicotinamide has a dual effect on the proliferation of HeLa cells. At 1mM proliferation was slightly increased whereas at 3mM inhibition was inhibited approximately 20% (96). 10mM 5-methylnicotinamide completely inhibits HeLa proliferation causing an accumulation of cells in G_2 phase (286). A clone was isolated which was able to divide at 70% of the control rate in the presence of 10mM 5-methylnicotinamide (287). Poly(ADP-ribose) synthetase from this resistant clone was slightly less sensitive to 5-methylnicotinamide inhibition. Recently Nduka & Shall reported the isolation of a 5-methylnicotinamide resistant clone of L1210 cells although no properties of poly(ADP-ribose) synthetase from these cells were given (288).

The relative insensitivity of L1210 cells to 3-aminobenzamide, which is a more potent inhibitor of poly(ADP-ribose) synthetase than the compounds described above, casts doubt as to whether the proliferative effects observed with other inhibitors are mediated via ADP-ribosylation of nuclear proteins.

4.3.7 Incorporation of radioactive precursors into acid-insoluble material

The effect of 2mM 3-aminobenzamide on precursor incorporation into macromolecules is shown in Figure 22. As might be expected from its lack of effect on cellular proliferation, the incorporation of [^{3}H]-thymidine is unaffected. Similarly, [^{3}H]-uridine incorporation does not vary

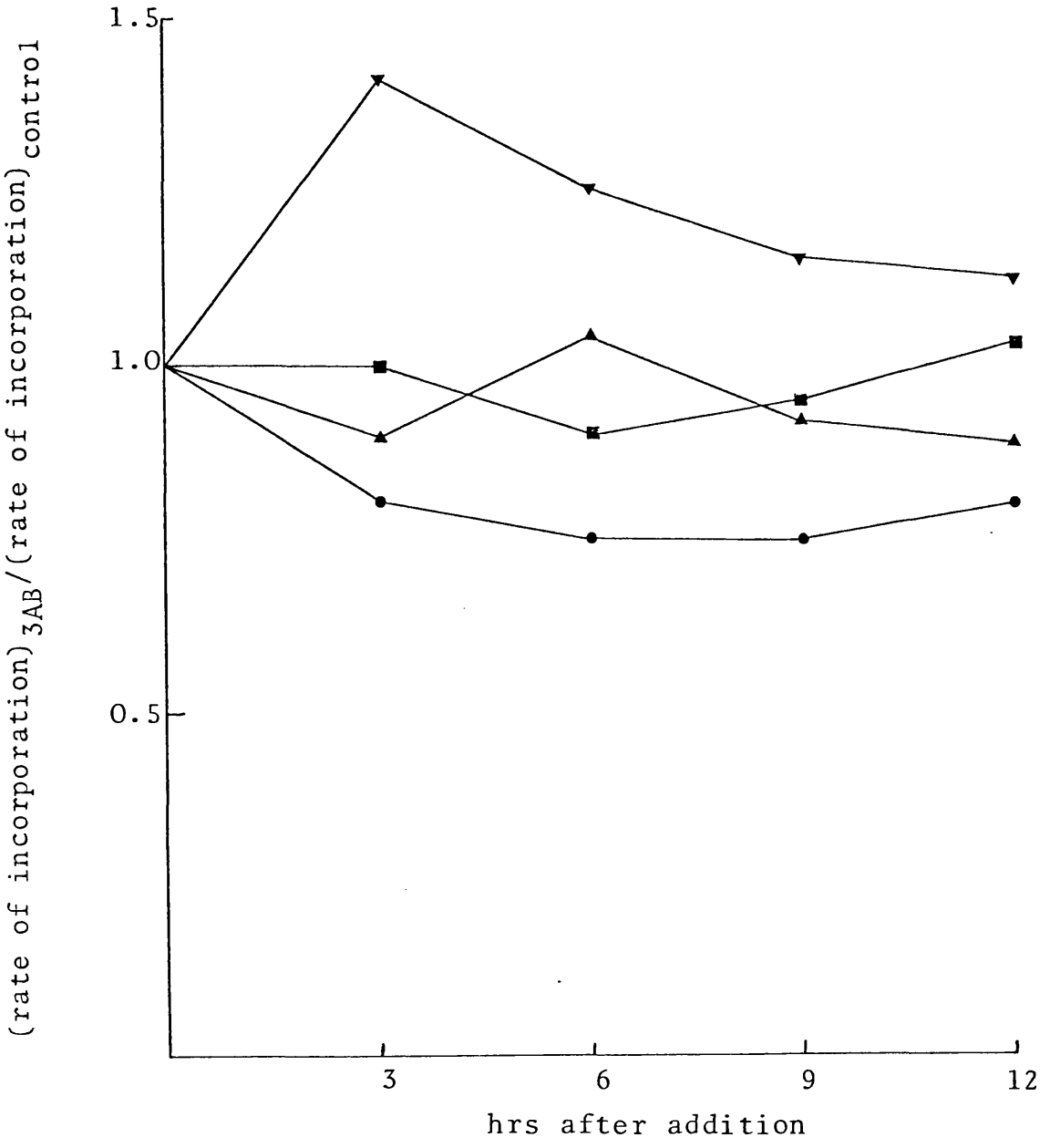


Figure 22 The effect of 2mM 3-aminobenzamide on incorporation of precursors into acid-insoluble material.

■, ^{3}H-thymidine; ▲, ^{3}H-uridine; ●, ^{3}H-adenosine;
▼, ^{3}H-leucine

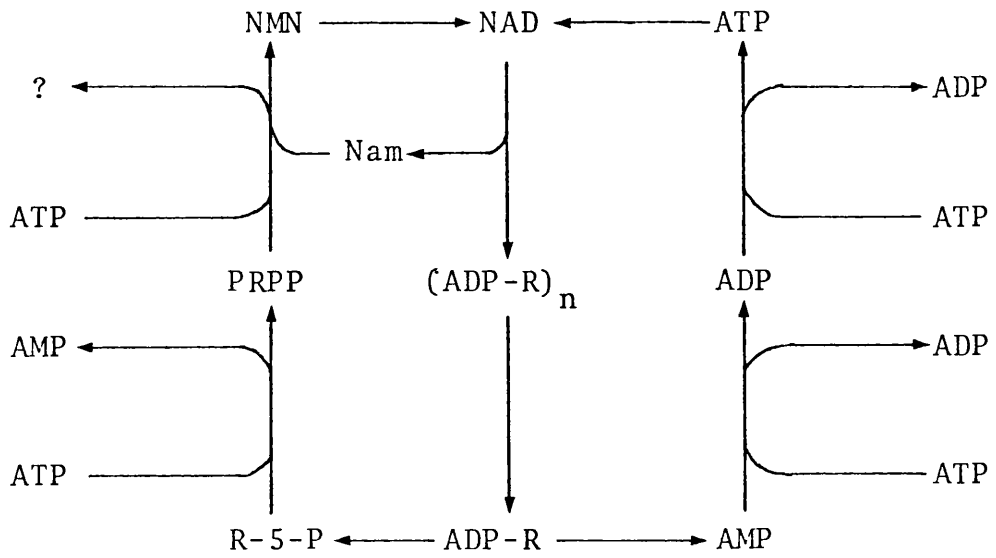


Figure 23 Schematic representation of NAD turnover in L1210 cells

significantly from control rates. These two observations rule out a direct regulatory role for ADP-ribosylation in either DNA replication or transcription in these cells.

It might be expected that adenosine incorporation would be unaffected also. The slight decrease in the rate of ^{3}H-adenosine incorporation in the presence of 2mM 3-aminobenzamide is unlikely to be as a result of a change in levels of ADP-ribosylated proteins because of the low levels *in vivo*. A more plausible explanation is that 3-aminobenzamide is affecting the turnover of ATP. The turnover of NAD via poly(ADP-ribose) synthetase involves the hydrolysis of 4-6 high energy phosphate bonds (it is uncertain whether ATP is hydrolysed during the synthesis of NMN from nicotinamide and phosphoribosyl pyrophosphate) as can be seen in Figure 23. Inhibition of this cycle might have a significant effect on the turnover of ATP if poly(ADP-ribose) synthetase is largely responsible for the short half life of NAD in eukaryotic cells.

The increase in leucine incorporation is puzzling. The lack of effect on uridine incorporation suggests that 3-amino benzamide is acting at a post-transcriptional level although a small increase in transcription might be masked. One possibility is that protein degradation is affected by 3-aminobenzamide directly or via ADP-ribosylation. Benzamide is known to inhibit chymotrypsin with a Ki of 10 mM (289). The peptidase activity of carboxypeptidase A is stimulated by 25 mM benzamide (290). 3-Aminobenzamide

might stimulate proteolysis if L1210 cells contain a protease similar to that isolated by Suzuki & Murachi from macrophages (see Introduction). A further possibility is that the transport of leucine into the cell is affected by 3-aminobenzamide.

Clearly further work is needed to elucidate the molecular mechanisms by which 3-aminobenzamide treatment increases [^{3}H]-leucine incorporation into proteins.

4.3.8 The effect of 2mM 3-aminobenzamide on NAD levels in L1210 cells

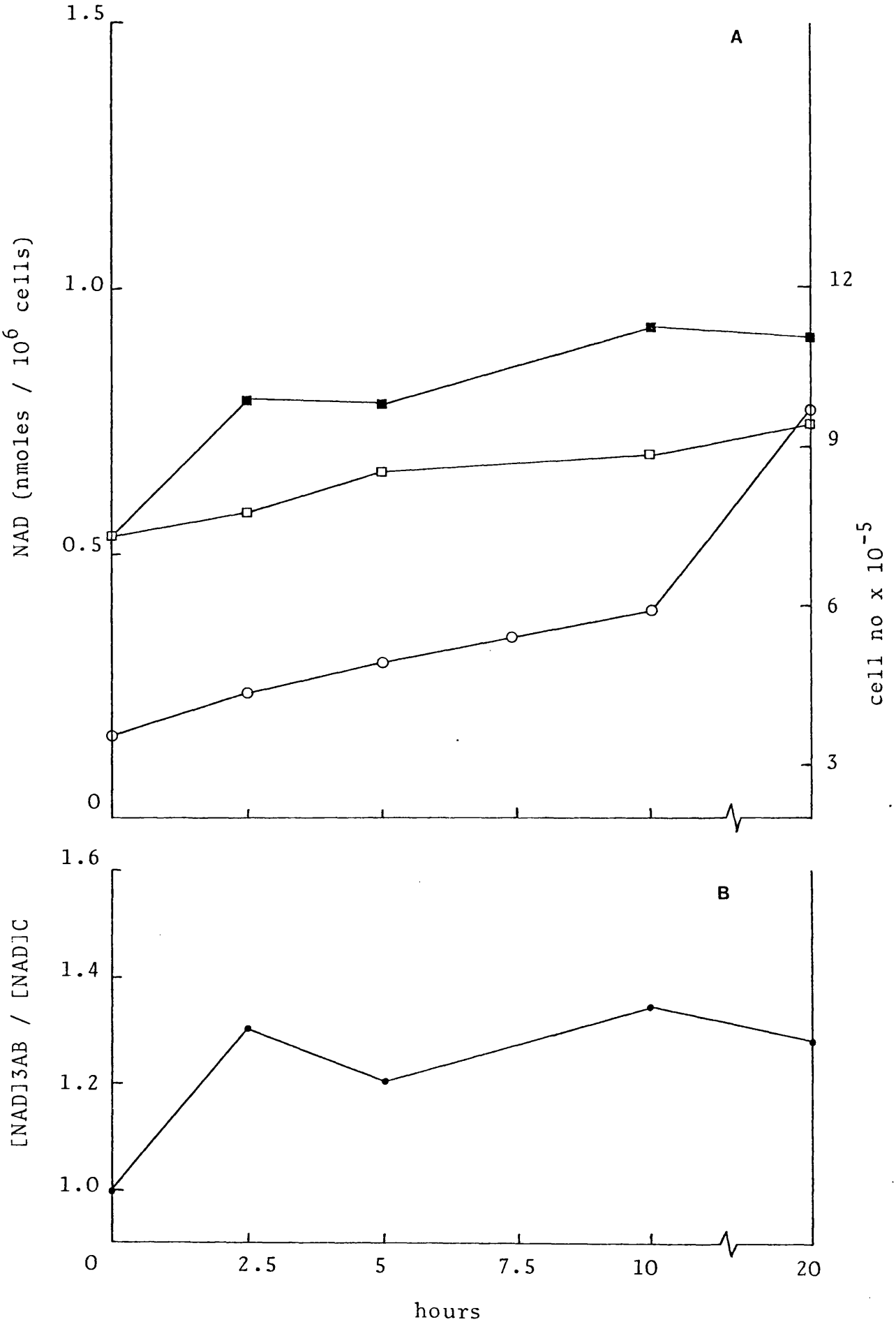
If poly(ADP-ribose) synthetase is a major degradation enzyme for NAD then treatment of cells with 3-aminobenzamide should result in an elevation of intracellular levels. An increase in NAD was found (Fig. 24). The concentration of pyridine nucleotides found in control cells is slightly lower than those reported for L1210 cells by Shall and co-workers (195,263). This probably represents $NADH_2$ which would be degraded under the assay conditions used (ie extraction with $HClO_4$). The increase observed with 3-aminobenzamide is not as great as might be expected from the half life of 1 hour reported for eukaryotic cells (146) although a similar increase is observed when L1210 cells are treated with 5-methylnicotinamide and a DNA damaging agent (236). Whether this means that poly(ADP-ribose) synthetase is not the major degradative enzyme for NAD or that NAD synthesis is decreased is uncertain at present. It may be significant

Figure 24 The effect of 2mM 3-aminobenzamide on NAD levels in L1210 cells.

A. The results from a single experiment.

○, cell number
□, NAD level in control cells
■, NAD level in 3-aminobenzamide treated cells

B. The mean ratio from four separate determinations.


A
B
NAD (nmoles / 10^6 cells)
cell no x 10^{-5}
[NAD]3AB / [NAD]C
hours
1.5
1.0
0.5
0
12
9
6
3
1.6
1.4
1.2
1.0
0
2.5
5
7.5
10
20

that NMN pyrophosphorylase from rat liver is inhibited by NAD and $NADH_2$ (279). Furthermore the supply of precursors may be a rate limiting step.

The main conclusion to be drawn from the elevated NAD levels is that although 2mM 3-aminobenzamide has no gross effect on cell proliferation it is inhibiting ADP-ribosylation *in vivo*.

4.3.9 Poly(ADP-ribose) synthetase activity in permeabilized cells following 3-aminobenzamide treatment

Tanuma *et al* (164) observed an increase in the activity of poly(ADP-ribose) synthetase in isolated nuclei following treatment of HeLa cells with thymidine and nicotinamide but not hydroxyurea or amethopterin. This was suggested to be a result of an increase in the number of acceptor proteins *in vitro* as a result of inhibition of ADP-ribosylation *in vivo*. In order to test whether a similar result could be obtained in L1210 cells after treatment with 3-aminobenzamide, the activity of poly(ADP-ribose) synthetase was determined in permeabilized cells after various times after the addition of 3-aminobenzamide to cultures. Contrary to the results of Tanuma *et al*, no significant difference in the activity was observed in permeabilized cells. Indeed after 25 hours the activity in treated cells was slightly decreased (Fig. 25). It is questionable whether the results in HeLa cells are due to an increase in acceptors. Using immunological techniques to assay levels *in vivo*, Kun's

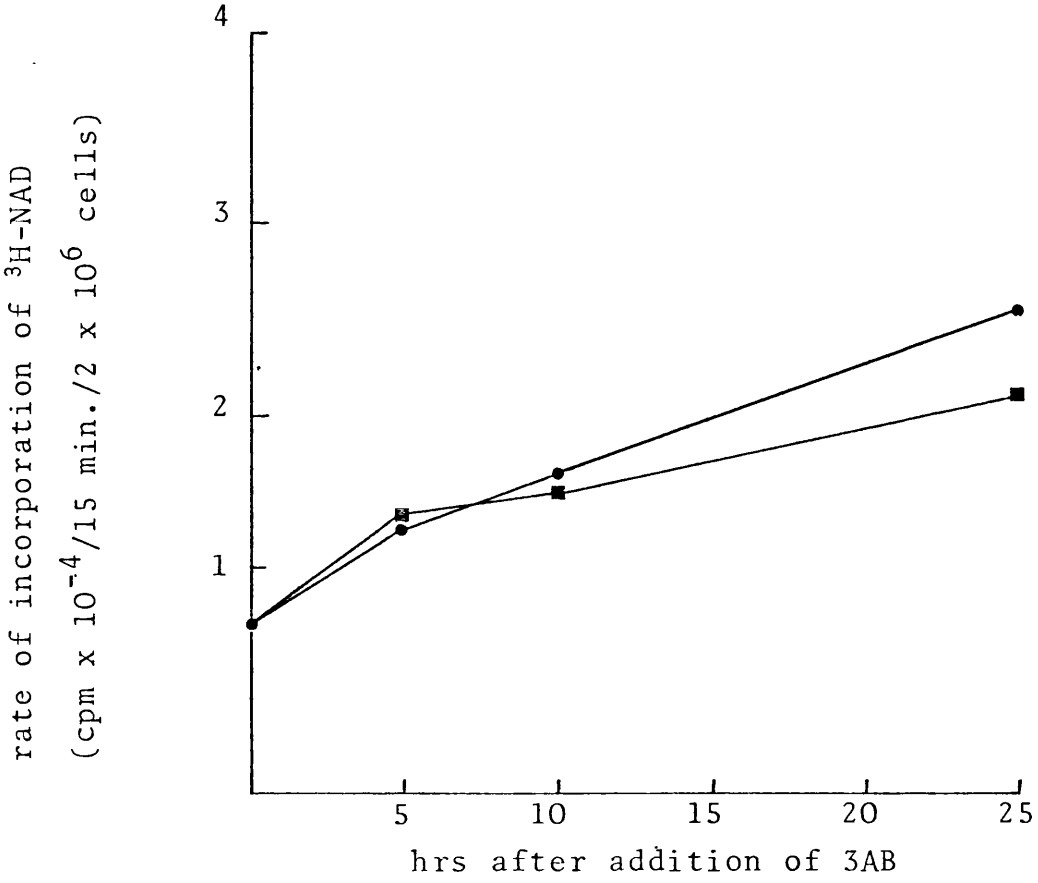


Figure 25 The effect of 2mM 3-aminobenzamide treatment *in vivo* on the activity of poly(ADP-ribose) synthetase in permeabilised cells.

●, control
■, 2mM 3AB

group (69) and Hilz's group (291) observed increases in poly(ADP-ribose) and mono-ADP-ribose protein conjugates respectively in rat liver following administration of nicotinamide. Furthermore, recently Kidwell et al have reported that treatment of HeLa cells with picolinic acid causes an increase in both poly(ADP-ribose) levels in vivo and in the activity of poly(ADP-ribose) synthetase in permeabilized cells (286). One attractive possibility for the difference between the results presented here and those of Tanuma et al is that treatment with inhibitors of poly(ADP-ribose) synthetase in vivo changes nuclear structure. This could result in differences in the state of the nuclei isolated from treated cells following cell disruption by homogenization. Evidence supporting this possibility comes from the data of Wielckins et al who found that although the activity of poly(ADP-ribose) synthetase was higher in CLL cells than control lymphocytes when assayed in homogenates, the initial activities in permeabilized cells were identical (162).

4.4. General Discussion

The lack of a gross effect on any of the parameters measured upon treatment of L1210 cells with 2mM 3-amino-benzamide is difficult to reconcile with the numerous regulatory functions ascribed to ADP-ribosylation of nuclear proteins. Possible explanations for this finding

are listed below:-

1. 3-Aminobenzamide does not inhibit ADP-ribosylation of nuclear proteins *in vivo*.
2. ADP-ribosylation is involved in the regulation of a function not investigated in the present study.
3. ADP-ribosylation has a regulatory function on one of the parameters investigated in normal cells but this regulation is absent from L1210 cells.
4. ADP-ribosylation represents a control mechanism but the cells can adapt to inhibition of poly(ADP-ribose) synthetase by another mechanism.

These are discussed in greater detail below.

1. Suitability of 3-aminobenzamide as a probe in vivo

Results presented earlier clearly show that 3-aminobenzamide is an extremely potent inhibitor of poly(ADP-ribose) synthetase *in vitro* as judged by the observed decrease in the incorporation of ^{3}H-NAD into acid-insoluble material by an extract from nuclei isolated from pig thymus (Section 3.3.1), by isolated nuclei from L1210 cells and by L1210 cells made permeable to nucleotides (Section 4.3.1). At the concentration of 2mM 3-aminobenzamide which was used for the bulk of the studies *in vivo*, the rate of ADP-ribosylation in permeable cells (which is reported to closely resemble ADP-ribosylation *in vivo*) was not measurable. The concentration of intracellular 3-aminobenzamide rapidly approaches that of the surrounding

medium. Even after 48 hours exposure, the bulk of the fluorescent material from inside the cells migrates with the same Rf as authentic 3-aminobenzamide when analysed by t.l.c. (Section 4.3.5). Thus inactivation of the inhibitor inside the cell can be discounted.

The increase in NAD levels in cells treated with 3-aminobenzamide is a good indication that poly(ADP-ribose) synthetase is inhibited *in vivo*. The increase could arise from either an increased rate of NAD biosynthesis or a decreased rate of NAD hydrolysis. A direct effect on NAD biosynthesis by 3-aminobenzamide can be discounted because such an effect would have been observed when NMN pyrophosphorylase or NAD pyrophosphorylase were assayed *in vitro*. The majority of NAD degradation in eukaryotic cells has been shown to occur in the nucleus and occur via hydrolysis of the nicotinamide-ribose linkage. This has prompted Reichsteiner *et al* to suggest that poly(ADP-ribose) synthetase is responsible for the bulk of NAD hydrolysis in the cell. A further indication that 3-aminobenzamide is active inside the cells is the greater inhibition of ^{3}H-adenosine incorporation into acid-insoluble material than either ^{3}H-thymidine or ^{3}H-uridine incorporation which suggests a perturbation of adenosine metabolism.

The major criticism of the present study is that no attempt has been made to show an alteration in levels of mono and poly(ADP-ribose)-protein conjugates inside the cell. With this reservation borne in mind, however, there

is no reason to suggest that 3-aminobenzamide is not inhibiting poly(ADP-ribose) synthetase *in vivo*.

2. ADP-ribosylation as a modulator of some other function

Of the functions ascribed to ADP-ribosylation by other workers in the field, an essential role in the regulation of DNA replication and transcription can be discounted. Furthermore, if ADP-ribosylation is involved in a different function, then the lack of an effect on cellular proliferation must be kept in mind. Although not examined in the present study, 3-aminobenzamide has been shown to inhibit the repair of DNA strand-breaks induced by the monofunctional alkylating agent, dimethyl sulphate (195). This, together with the data reported in the Introduction (Section 1.3.3), suggests that ADP-ribosylation is involved in the repair of DNA.

There has been some controversy as to whether a process resembling DNA repair occurs in cells which have not been exposed to a DNA damaging agent. In addition to reports showing incorporation of ^{3}H-thymidine into cells which were not undergoing semi-conservative DNA replication (292), Painter & Young (293) found significant incorporation of ^{3}H-bromodeoxyuridine into parental strand DNA after denaturation of parental and daughter strand DNA with heat and formaldehyde with subsequent separation of the strands using density gradient centrifugation. If however separation was performed under alkaline conditions, no such

incorporation was observed (294). From this it was concluded that the use of heat and formaldehyde to denature parental and daughter DNA strands followed by centrifugation under neutral conditions led to limited renaturation of the two strands. Smith and Hanawalt (295), who also performed centrifugation under alkaline conditions, also concluded that DNA repair did not occur in unirradiated cells. The criterion of base incorporation into parental strand DNA does not preclude the excision of mismatched base pairs from daughter strand DNA after the cessation of semi-conservative DNA replication. Such a process could account for the observed increase in poly(ADP-ribose) levels *in vivo* during the G_2 phase of the cell cycle.

Although inhibition of such a process might not have an immediate effect on cell proliferation, it is uncertain whether such a process could account for the high rate of NAD turnover in eukaryotic cells. Strayer & Boyer (296) concluded that, in E.coli, the parental DNA strand is subject to a high rate of phosphodiester backbone breakage and ligation during replication from the frequency of incorporation of thymidine (1 per 10,000-20,000 residues), phosphate (1 per 7,000 residues) and ^{18}O, derived from $H_2{}^{18}O$, into phosphate (1 per 100-500 residues). A high rate of phosphodiester backbone breakage/ligation would relieve the strain produced by unwinding of the two DNA strands during replication and possibly transcription. Although the data was obtained from E.coli, which has not been found to contain poly(ADP-ribose) synthetase, the existence of a

similar process in eukaryotic cells could be inferred from the finding of Hilton & Walker (297) that depletion of ATP, necessary for the ligation step, leads to an accumulation of single strand breaks in the DNA of L1210 and HeLa cells. An involvement in the regulation of endonucleolytic activity is an attractive role for ADP-ribosylation in view of the observed modulation of endonuclease activity by ADP-ribosylation (54,298,299). The increased rate of sister chromatid exchanges induced by nicotinamide (300) and the increase in chromosomal abnormalities in primary mongoose kidney cultures upon treatment with benzamide (301) is also consistent with the involvement of ADP-ribosylation in endonucleolytic activity.

3. L1210 cells as a model for the study of ADP-ribosylation in vivo

Although L1210 cells are attractive as a model system in terms of the ease with which they can be manipulated, their very nature as cancer cells may present difficulties. ADP-ribosylation of nuclear proteins may represent a regulatory mechanism in normal cells but the process of transformation may have by-passed such a mechanism. Sugimura's group has observed differences in gross levels of poly(ADP-ribose) synthetase activity and the activity through the growth cycle when SV40 transformed cell lines are compared to non-transformed cell lines (154). Further evidence for such a possibility is that proliferation of human lymphocytes following treatment with phytohaemagglutinin

is inhibited at high levels of nicotinamide (302).

The observation that inhibitors of poly(ADP-ribose) synthetase induce differentiation of Friend cells (215,303) and inhibit the differentiation of chick embryo myoblasts (304) suggests that poly(ADP-ribose) synthetase may be involved in the process of gene expression. If fluctuations in the turnover or gross levels of ADP-ribosylated proteins only serves to activate or repress certain genes, but not to maintain their transcription, and if these genes are switched on or off in L1210 cells, then inhibition of poly(ADP-sibose) synthetase would not affect their growth. Alternatively if only a small number of genes are regulated by ADP-ribosylation then their activation or repression by ADP-ribosylation would not be detected by the incorporation of precursors into macromolecules.

4. Adaptation by other processes to inhibition of ADP-ribosylation

To discuss such a proposition, one should consider the ways in which ADP-ribosylation could exert an influence and then examine alternative mechanisms by which the cell could achieve a similar end.

(a) Inhibition of an enzyme

If ADP-ribosylation exerts its action by either direct modification of an enzyme's active site or by a non-covalent mechanism, then treatment with 3-aminobenzamide would be expected to increase the activity. The activity could be

decreased by enhanced proteolytic degradation since poly(ADP-ribose) is known to inhibit a nuclear protease. Circumstantial evidence supports such a hypothesis in the case of RNA polymerase 1. As stated in the Introduction, RNA polymerase 1 is thought to be inhibited by ADP-ribosylation. In rat liver, it has been shown that RNA polymerase 1 has an extremely short half life (305). Furthermore treatment of rats with hydrocortisone or isobutylmethylxanthine, both of which decrease poly(ADP-ribose) synthetase activity (306,99), results in a transient stimulation of RNA polymerase 1 activity and this enhanced activity is extremely sensitive to proteolytic digestion (307).

The possibility that free, rather than protein bound, poly(ADP-ribose) may be important in the regulation of cellular function is suggested by the finding that free poly(ADP-ribose) is able to induce the differentiation of mouse myeloid leukaemic cells into macrophages or granulocytes (216).

(b) Accessibility of DNA to enzymes

If ADP-ribosylation of nuclear proteins serves to decrease their affinity for DNA and thereby exposing the DNA to enzymatic attack, inhibition of poly(ADP-ribose) synthetase would result in tighter binding to DNA. This may be compensated for by enhanced proteolysis as a result of lowered poly(ADP-ribose) levels (see above).

(c) Chromatin condensation

The observation that administration of inhibitors of poly(ADP-ribose) synthetase to rats results in a rise in the activity of ornithine decarboxylase in liver and brain raises the possibility that decreased chromatin condensation, normally regulated by ADP-ribosylation, may be compensated for by an increase in polyamine levels.

SECTION 5

CONCLUSIONS

5. CONCLUSIONS

The objective of this work was to find a physiologically specific inhibitor of poly(ADP-ribose) synthetase and use it as a probe *in vivo* to determine the function of ADP-ribosylation. The project was a complete success in that one of the new compounds screened viz. 3-aminobenzamide was shown to be not only more specific in its action than any previously described inhibitor but it, together with a variety of other benzamides, was also a much more potent inhibitor than those used previously.

Though the data obtained from studies *in vivo* did not reveal the exact function of ADP-ribosylation valuable insight into the possible biological roles of protein ADP-ribosylation was obtained. It was shown that 3-aminobenzamide was able to enter L1210 cells at concentrations sufficient to completely inhibit ADP-ribosylation in permeablized cells. Treatment of L1210 cells with 2 mM 3-aminobenzamide had no observable effect on parameters such as the rate of proliferation and incorporation of radioactive thymidine or uridine into macromolecules. Incorporation of radioactive leucine was transiently stimulated. The finding that ^{3}H -adenosine incorporation was slightly inhibited can be explained by a decrease in the rate of ATP turnover as a consequence of inhibition of NAD degradation via poly(ADP-ribose) synthetase. The increase in intracellular NAD levels following 3-aminobenzamide treatment supports such an explanation.

Despite the lack of positive results from studies *in vivo*, the information obtained is valuable in that ADP-ribosylation cannot have a major regulatory role in processes such as cell proliferation, the cell cycle, DNA replication and DNA transcription.

The role of ADP-ribosylation cannot be elucidated from the findings presented here but data from Shall's lab has shown that 3-aminobenzamide inhibits DNA repair and myoblast differentiation (195, 304). Thus, in conclusion, the use of 3-aminobenzamide as an inhibitor of poly(ADP-ribose) synthetase has already provided valuable information as to the role of ADP-ribosylation and will almost certainly yield more in the future.

SECTION 6

REFERENCES

REFERENCES

1. Hilz, H. & Stone, P.R. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1-58.

2. Bodley, J.W., Van Ness, B.G. & Howard, J.B. (1980) Dev. Cell. Biol. 6, 413-424.

3. Moss, J. & Vaughan, M. (1979) Annu. Rev. Biochem. 48, 581-600.

4. Gill, D.M. & Meren, R. (1978) Proc. Natl. Acad. Sci., USA. 75, 3050-3054.

5. Rodbell, M. (1980) Nature (London) 284, 17-22.

6. Oppenheimer, N.J. (1978) J. Biol. Chem. 253, 4907-4910.

7. Goff, C.G. (1974) J. Biol. Chem. 249, 6181-6190.

8. Rohrer, H., Zillig, W. & Mailhammer, R. (1975) Eur. J. Biochem. 60, 227-238.

9. Skorko, R., Zillig, W., Rohrer, H., Fujiki, H. & Mailhammer, R. (1977) Eur. J. Biochem. 79, 55-66.

10. Pesce, A., Casoli, C. & Schito, G.C. (1976) Nature (London) 262, 412-414.

11. Chambon, P., Weill, J.D., Doly, J., Strosser, M.T. & Mandel, P. (1966) Biochem. Biophys. Res. Comm. 25, 638-643.

12. Reeder, R.H., Ueda, K., Honjo, T., Nishizuka, Y. & Hayaishi, O. (1967) J. Biol. Chem. 242, 3172-3179.

13. Fujimura, S., Hasegawa, S. & Sugimura, T. (1967) Biochem. Biophys. Acta 134, 496-499.

14. Chambon, P., Weill, J.D. & Mandel, P. (1963) Biochem. Biophys. Res. Comm. 11, 29-43.

15. Sugimura, T. (1973) Prog. Nuc. Acids Res. Mol. Biol. 13, 127-151.

16. Hayaishi, O. & Ueda, K. (1977) Annu. Rev. Biochem. 46, 95-116.

17. Adamietz, P. & Hilz, H. (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 527-534.

18. Bredehorst, R., Wielckins, K., Gartemann, A., Lengyel, H., Klapproth, K. & Hilz, H. (1978) Eur. J. Biochem. 92, 129-135.

19. Adamietz, P., Bredehorst, R. & Hilz, H. (1978) Eur. J. Biochem. 91, 317-326.

20. Nishizuka, Y., Ueda, K., Nakazawa, K. & Hayaishi, O. (1967) J. Biol. Chem. 242, 3164-3171.

21. Nishizuka, Y., Ueda, K., Honjo, T. & Hayaishi, O. (1968) J. Biol. Chem. 243, 3765-3767.

22. Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M. & Hayaishi, O. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 781-786.

23. Kun, E., Chang, A.C.Y., Sharma, M.L., Ferro, A.M. & Nitecki, D. (1976) Proc. Natl. Acad. Sci., USA. 73, 3131-3135.

24. Smith, J.A. & Stocken, L.A. (1973) Biochem. Biophys. Res. Comm. 54, 297-300.

25. Smith, J.A. & Stocken, L.A. (1975) Biochem. J. 147, 523-529.

26. Burzio, L.O., Riquelme, P.T. & Koide, S.S. (1979) J. Biol. Chem. 254, 3029-3037.

27. Riquelme, P.T., Burzio, L.O. & Koide, S.S. (1979) J. Biol. Chem. 254, 3018-3028.

28. Hayaishi, O., Ueda, K., Kawaichi, M., Ogata, N., Kiai, K., Oka, J., Kim, H.R., Ito, S., Shizuta, Y. & Okayama, H. (1979) Fed. Proc. 38, 618.

29. Ogata, N., Ueda, K. & Hayaishi, O. (1980) Dev. Cell Biol. 6, 333-344.

30. Miwa, M., Saiko, H., Sakura, H., Saikawa, N., Watanabe, F., Matsushima, T. & Sugimura, T. (1977) Nuc. Acids Res. 4, 3997-4005.

31. Ferro, A.M. & Oppenheimer, N.J. (1978) Proc. Natl. Acad. Sci., USA. 75, 809-813.

32. Inagaki, F., Miyazawa, T., Miwa, M., Saito, H. & Sugimura, T. (1978) Biochem. Biophys. Res. Comm. 85, 415-420.

33. Sugimura, T., Yoshimura, N., Miwa, M., Nagai, H. & Nagao, M. (1971) Arch. Biochem. Biophys. 147, 660-665.

34. Tanaka, M., Miwa, M., Hayashi, K., Kubota, K., Matsushima, T. & Sugimura, T. (1977) Biochemistry 16, 1485-1489.

35. Adamietz, P., Bredehorst, R. & Hilz, H. (1978) Biochem. Biophys. Res. Comm. 81, 1377-1383.

36. Tanaka, M., Hayashi, K., Sakura, H., Miwa, M., Matsushima, T. & Sugimura, T. (1978) Nuc. Acids Res. 5, 3183-3194.

37. Miwa, M., Saikawa, M., Yamaizumi, Z., Nishimura, S. & Sugimura, T. (1979) Proc. Natl. Acad. Sci. USA. 76, 595-599.

38. Miwa, M., Ishihara, M., Takashima, S., Saikawa, N., Yamaizumi, Z., Nishimura, S. & Sugimura, T. (1980) Dev. Cell Biol. 6, 1-10.

39. Farzaneh, F. & Pearson, C.K. (1978) Biochem. Biophys. Res. Comm. 84, 537-543.

40. Furneaux, H.M. & Pearson, C.K. (1980) Biochem. J. 187, 91-103.

41. Rickwood, D., MacGillivray, A.J. & Whish, W.J.D. (1977) Eur. J. Biochem. 79, 589-598.

42. Wong, N.C., Dixon, G.H. & Poirier, G. (1977) Eur. J. Biochem. 77, 11-21.

43. Lichtenwalner, D.M. & Suhadolnik, R.J. (1979) Biochemistry 18, 3749-3755.

44. Okayama, H., Ueda, K. & Hayaishi, O. (1978) Proc. Natl. Acad. Sci. USA. 75, 1111-1115.

45. Okayama, H. & Hayaishi, O. (1978) Biochem. Biophys. Res. Comm. 84, 755-762.

46. Whitby, A.J., Stone, P.R. & Whish, W.J.D. (1979) Biochem. Biophys. Res. Comm. 90, 1295-1304.

47. Willmitzer, L. (1979) FEBS Lett. 108, 13-16.

48. Tanuma, S., Enomoto, T. & Yamada, M. (1977) Biochem. Biophys. Res. Comm. 74, 599-605.

49. Stone, P.R., Lorimer, III, W.S. & Kidwell, W.R. (1977) Eur. J. Biochem. 81, 9-18.

50. Jump, D.B., Butt, T.R. & Smulson, M.E. (1979) Biochemistry 18, 983-990.

51. Adamietz, P., Bredehorst, R. & Hilz, H. (1978) Eur. J. Biochem. 91, 317-326.

52. Lorimer, III, W.S., Stone, P.R. & Kidwell, W.R. (1977) Exp. Cell Res. 106, 261-266.

53. Adamietz, P., Klapproth, K. & Hilz, H. (1979) Biochem. Biophys. Res. Comm. 91, 1232-1238.

54. Yoshihara, K., Tanigawa, Y., Burzio, L.O. & Koide, S.S. (1975) Proc. Natl. Acad. Sci. USA. 72, 289-293.

55. Yoshihara, K., Hashida, T., Yoshihara, H., Tanaka, Y. & Oligushi, H. (1977) Biochem. Biophys. Res. Comm. 59, 300-306.

56. Caplan, A.I., Niedergang, C., Okazaki, H. & Mandel, P. (1979) Arch. Biochem. Biophys. 198, 60-69.

57. Okayama, H., Edson, D.M., Fukushima, M., Ueda, K. & Hayaishi, O. (1977) J. Biol. Chem. 252, 7000-7005.

58. Ellison, R.P. (1978) J. Cell Biol. 79, 131a.

59. Ueda, K., Omachi, A., Kawaichi, M. & Hayaishi, O. (1975) Proc. Natl. Acad. Sci. USA. 72, 205-209.

60. Ord, M.G. & Stocken, L.A. (1977) Biochem. J. 161, 583-592.

61. Minaga, T., Romaschin, A.D., Kirsten, E. & Kun, E. (1979) J. Biol. Chem. 254, 9663-9668.

62. Stone, P.R., Bredehorst, R., Kittler, M., Lengyuel, H. & Hilz, H. (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 51-56.

63. Niedergang, C., Okazaki, H. & Mandel, P. (1978) Anal. Biochem. 88, 20-28.

64. Sims, J.L., Juarez, H. & Jacobson, M.K. (1979) Fed. Proc. 38, 493.

65. Juarez-Salinas, H., Sims, J.L. & Jacobson, M.K. (1979) Nature (London) 282, 740-741.

66. Kanai, Y., Miwa, M., Matsushima, T. & Sugimura, T. (1974) Biochem. Biophys. Res. Comm. 59, 300-306.

67. Sakura, H., Miwa, M., Tanaka, M., Kanai, Y., Shimada, T., Matsushima, T. & Sugimura, T. (1977) Nuc. Acids Res. 4, 2903-2915.

68. Kidwell, W.R. & Mage, M. (1976) Biochemistry 15, 1213-1217.

69. Ferro, A.M., Minaga, T., Piper, W.N. & Kun, E. (1978) Biochim. Biophys. Acta 519, 291-305.

70. Ikai, K., Ueda, K. & Hayaishi, O. (1980) Dev. Cell Biol. 6, 173-184.

71. Sakura, H., Miwa, M., Kanai, Y., Matsushima, T. & Sugimura, T. (1978) Nuc. Acids Res. 5, 4025-4033.

72. Ikejima, M., Sakura, H., Miwa, M., Kanai, Y., Sezawa, K. & Sugimura, T. (1980) Dev. Cell Biol. 6, 165-172.

73. Kanai, Y., Sugimura, T. & Matsushima, T. (1978) Nature (London) 274, 809-812.

74. Kanai, Y., Sugimura, T., Aotsuka, S. & Yokohari, R. (1980) Dev. Cell Biol. 6, 153-164.

75. Kanai, Y., Kawaminami, Y., Miwa, M., Matsushima, T., Sugimura, T., Morai, Y. & Yokohari, R. (1977) Nature (London) 265, 175-177.

76. Okolie, E.E. & Shall, S. (1979) Clin. Exp. Immunol. 36, 151-164.

77. Stone, P.R. & Hilz, H. (1975) FEBS Lett. 57, 209-212.

78. Goebel, M., Stone, P.R., Lengyel, H. & Hilz, H. (1977) Hoppe Seyler's Z. Physiol. Chem. 358, 13-21.

79. Bredehorst, R., Ferro, A.M. & Hilz, H. (1978) Eur. J. Biochem. 82, 105-113.

80. Bredehorst, R., Ferro, A.M. & Hilz, H. (1978) Eur. J. Biochem. 82, 115-121.

81. Stocken, L.A. (1978) Methods in Cell Biology XIX, 167-174.

82. Kun, E., Zimber, P.H., Chang, A.C.Y., Puschendorf, B. & Grunicke, H. (1975) Proc. Natl. Acad. Sci. USA. 72, 1436-1440.

83. Burzio, L.O., Luke, M. & Koide, S.S. (1979) Fed. Proc. 38, 618.

84. Roberts, J.H., Stark, P., Giri, C.P. & Smulson, M.E. (1975) Arch. Biochem. Biophys. 171, 305-315.

85. Brightwell, M.D., Leech, C.E., O'Farrell, M.K., Whish, W.J.D. & Shall, S. (1975) Biochem. J. 147, 119-129.

86. Smulson, M.E., Henrikson, O. & Rideau, C. (1971) Biochem. Biophys. Res. Comm. 43, 1266-1273.

87. Suhadolnik, R.J., Baur, R.J., Lichtenwalner, D.M., Uematsu, T., Roberts, J.H., Sudhakar, S.K. & Smulson, M.E. (1977) J. Biol. Chem. 252, 4134-4144.

88. Farina, B., Rosaria, M., Mennella, F. & Mauro, A. (1980) Rend. Acad. Sci. Fis. Mat., Naples 46, 359-363.

89. Miller, E.G. (1975) Biochim. Biophys. Acta 395, 191-200.

90. Miller, E.G. (1975) Biochem. Biophys. Res. Comm. 66, 280-286.

91. Benjamin, R.C. & Gill, D.M. (1978) J. Supramol. Str., supp. 2, 74.

92. Whitby, A.J. (1980) Ph.D. Thesis, University of Bath, UK.

93. Poirier, G.G., Savarel, P., Rojotte, D., Morrisset, J. & Lord, A. (1978) Can. J. Biochem. 56, 784-790.

94. Kidwell, W.R. & Burdette, K.E. (1974) Biochem. Biophys. Res. Comm. 61, 766-773.

95. Dietrich, L.S. & Siebert, G. (1973) Hoppe Seyler's Z. Physiol. Chem. 354, 1133-1140.

96. Preiss, J., Schlaeger, R. & Hilz, H. (1971) FEBS Lett. 19, 244-246.

97. Clark, J.B., Ferris, G.M. & Pinder, S. (1971) Biochim. Biophys. Acta 238, 82-85.

98. Shall, S. (1975) J. Biochem. (Tokyo) 77, 2p.

99. Levi, V., Jacobson, E.L. & Jacobson, M.K. (1978) FEBS Lett. 88, 144-146.

100. Muller, W.E.G. & Zahn, R.K. (1976) Experientia 31, 1014-1015.

101. Tanigawa, Y., Kawamura, M. & Shimoyama, M. (1977) Biochem. Biophys. Res. Comm. 76, 406-412.

102. Perella, F.W. & Lea, M.A. (1978) Biochem. Biophys. Res. Comm. 82, 575-581.

103. Perella, F.W. & Lea, M.A. (1979) Cancer Res. 39, 1382-1389.

104. Khan, M.G. & Shall, S. (1976) Biochem. Soc. Trans. 4, 778-781.

105. Tsopanakis, C., MacLaren, E.A. & Shall, S. (1976) Biochem. Soc. Trans. 4, 774-777.

106. Tsopanakis, C., MacLaren, E.A., Tsopanakis, A. & Shall, S. (1977) Biochem. Soc. Trans. 5, 932-936.

107. Tsopanakis, C., MacLaren, E.A., Tsopanakis, A. & Shall, S. (1978) Eur. J. Biochem. 90, 337-345.

108. Okazaki, H., Niedergang, C. & Mandel, P. (1976) FEBS Lett. 62, 255-258.

109. Mandel, P., Okazaki, H. & Niedergang, C. (1977) FEBS Lett. 84, 331-336.

110. Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H. & Kamiya, T. (1978) J. Biol. Chem. 253, 6459-6466.

111. Ito, S., Shizuta, Y. & Hayaishi, O. (1979) J. Biol. Chem. 254, 3647-3651.

112. Kristensen, T. & Holtlund, J. (1976) Eur. J. Biochem. 70, 441-446.

113. Kristensen, T. & Holtlund, J. (1978) Eur. J. Biochem. 88, 495-501.

114. Holtlund, J., Kristensen, T. & Sletten, K. (1980) Biochem. J. 185, 779-782.

115. Jump, D.B. & Smulson, M.E. (1980) Biochemistry 19, 1024-1030.

116. Hashida, T., Ohgushi, H. & Yoshihara, K. (1979) Biochem. Biophys. Res. Comm. 88, 305-311.

117. Niedergang, C., Okazaki, H. & Mandel, P. (1979) Eur. J. Biochem. 102, 43-57.

118. Nolan, N. (1978) Ph.D. Thesis, The Catholic University of America, Washington D.C.

119. Ueda, K., Kawaichi, M., Okayama, H. & Hayaishi, O. (1979) J. Biol. Chem. 254, 679-687.

120. Kawaichi, M., Ueda, K. & Hayaishi, O. (1980) J. Biol. Chem. 255, 816-819.

121. Okazaki, H., Niedergang, C., Couppez, M., Martinage, A., Sautiere, P. & Mandel, P. (1980) FEBS Lett. 110, 227-229.

122. Tanaka, Y., Hashida, T., Yoshihara, H. & Yoshihara, K. (1979) J. Biol. Chem. 254, 12433-12438.

123. Halldorrson, H., Gray, D.A. & Shall, S. (1978) FEBS Lett. 85, 349-352.

124. Berger, N.A. & Johnson, E.S. (1976) Biochim. Biophys. Acta 425, 1-17.

125. Berger, N.A., Weber, G. & Kaichi, A.S. (1978) Biochim. Biophys. Acta 519, 87-104.

126. Futai, M., Mizuno, D. & Sugimura, T. (1967) Biochem. Biophys. Res. Comm. 28, 395-399.

127. Futai, M., Mizuno, D. & Sugimura, T. (1968) J. Biol. Chem. 243, 6325-6329.

128. Matsubara, H., Hasegawa, S., Fujimura, S., Shima, T., Sugimura, T. & Futai, M. (1970) J. Biol. Chem. 245, 3606-3611.

129. Shinsi, H., Miwa, M., Kato, K., Naguchi, M., Matsushima, T. & Sugimura, T. (1976) Biochemistry 15, 2185-2190.

130. Miwa, M., Tanaka, M., Shinsi, H., Takeuchi, M., Matsushima, T., Sugimura, T. & Shall, S. (1975) J. Biochem. (Tokyo) 77, 3p.

131. Hasegawa, S., Fujimura, S., Shimuzu, Y. & Sugimura, T. (1967) Biochim. Biophys. Acta 149, 369-376.

132. Miwa, M. & Sugimura, T. (1971) J. Biol. Chem. 246, 6362-6364.

133. Miwa, M., Tanaka, M., Matsushima, T. & Sugimura, T. (1974) J. Biol. Chem. 249, 3475-3482.

134. Ueda, K., Oka, J., Narumiya, S., Miyakawa, N. & Hayaishi, O. (1972) Biochem. Biophys. Res. Comm. 46, 516-523.

135. Miyakawa, N., Ueda, K. & Hayaishi, O. (1972) Biochem. Biophys. Res. Comm. 49, 239-245.

136. Stone, P.R., Whish, W.J.D. & Shall, S. (1973) FEBS Lett. 36, 334-337.

137. Tanaka, M., Miwa, M., Matsushima, T., Sugimura, T. & Shall, S. (1976) Arch. Biochem. Biophys. 172, 224-229.

138. Whitby, A.J. & Whish, W.J.D. (1978) Biochem. Soc. Trans. 6, 619-620.

139. Burzio, L.O., Riquelme, P.T., Ohtsuka, E. & Koide, S.S. (1976) Arch. Biochem. Biophys. 173, 306-319.

140. Miwa, M., Nakatsugawa, K., Hara, K., Matsushima, T. & Sugimura, T. (1975) Arch. Biochem. Biophys. 167, 54-60.

141. Stone, P.R., Lorimer, III, W.S., Ranchalis, J., Danley, M. & Kidwell, W.R. (1978) Nuc. Acids Res. 5, 173-184.

142. Okayama, H., Honda, M. & Hayaishi, O. (1978) Proc. Natl. Acad. Sci. USA. 75, 2254-2257.

143. Ueda, K., Kawaichi, M., Oka, J. & Hayaishi, O. (1980) Dev. Cell Biol. 6, 47-58.

144. Hogeboom, G. & Schneider, W. (1952) J. Biol. Chem. 197, 611-620.

145. Morton, R.K. (1958) Nature (London) 181, 540-542.

146. Reichsteiner, M., Hillyard, D. & Olivera, B.M. (1976) Nature (London) 259, 695-696.

147. Reichsteiner, M. & Catanzarite, V. (1974) J. Cell Physiol. 84, 409-422.

148. Olivera, B.M., Reichsteiner, M.C., Manlapaz Ramos, P., Cruz, L.J. & Hillyard, D. (1980) Dev. Cell Biol. 6, 85-98.

149. Burzio, L.O. & Koide, S.S. (1970) Biochem. Biophys. Res. Comm. 40, 1013-1020.

150. Janakidevi, K. (1978) Exp. Cell. Res. 112, 345-351.

151. Tanigawa, Y., Kawamura, N., Kitamura, A. & Shimoyama, M. (1978) Biochem. Biophys. Res. Comm. 81, 1278-1285.

152. Tanigawa, Y., Kitamura, N. & Shimoyama, M. (1978) Eur. J. Biochem. 92, 261-269.

153. Kitamura, N., Tanigawa, Y., Yamamoto, T., Kawamura, N., Doi, S. & Shimoyama, M. (1979) Biochem. Biophys. Res. Comm. 87, 725-733.

154. Miwa, M., Oka, K., Segawa, K., Tanaka, M., Ine, S., Yamaguchi, N., Kuchina, T., Shiroki, K., Shimojo, H., Sakura, H., Matsushima, T. & Sugimura, T. (1977) Arch. Biochem. Biophys. 181, 313-321.

155. Muller, W.E.G., Falke, D., Zahn, R.K. & Arendes, J. (1979) Intervirology 11, 182-187.

156. Ghani, Q.P. & Hollenberg, M. (1978) Biochem. J. 170, 387-394.

157. Ghani, Q.P. & Hollenberg, M. (1978) Biochem. Biophys. Res. Comm. 81, 886-891.

158. Stone, P.R. & Shall, S. (1975) Exp. Cell. Res. 91, 95-100.

159. Berger, N.A., Weber, G., Kaichi, A.S. & Petzold, S.J. (1978) Biochim. Biophys. Acta 519, 105-107.

160. Berger, N.A., Adams, J.W., Sikorski, G.W., Petzold, S.J. & Shearer, W.T. (1978) J. Clin. Invest. 62, 111-118.

161. Lehmann, A.R., Kirk-Bell, S. & Shall, S. (1973) Biochem. Soc. Trans. 1, 694.

162. Wielckins, K., Garbrecht, M., Kittler, M. & Hilz, H. (1980) Eur. J. Biochem. 104, 279-287.

163. Burzio, L.O., Reich, L. & Koide, S.S. (1975) Proc. Soc. Exp. Biol. Med. 149, 933-938.

164. Tanuma, S., Enomoto, T. & Yamada, M. (1979) J. Biol. Chem. 254, 4960-4962.

165. Bredehorst, R., Goebel, M., Renzi, F., Kittler, M., Klapproth, K. & Hilz, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1737-1743.

166. Burzio, L.O. & Koide, S.S. (1971) Biochem. Biophys. Res. Comm. 42, 1185-1190.

167. Furneaux, H.M. & Pearson, C.K. (1978) Biochem. Soc. Trans. 6, 753-755.

168. Muller, W.E.G. & Zahn, R.K. (1976) Mol. Cell. Biochem. 12, 147-159.

169. Muller, W.E.G., Totsuka, A., Nusser, I., Obermeier, J., Rhode, H. & Zahn, R.K. (1974) Nuc. Acids Res. 1, 1317-1327.

170. Hilz, H. & Kittler, M. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1693-1704.

171. Mullins, D.W., Giri, C.P. & Smulson, M.E. (1977) Biochemistry 16, 506-513.

172. Yukioka, M., Oka, Y., Hasuma, T. & Inoue, A. (1978) FEBS Lett. 86, 85-88.

173. Tsopanakis, C., Leer, J.C., Nielson, O.F., Gocke, E., Shall, S. & Westergaard, O. (1978) FEBS Lett. 93, 297-300.

174. Kawashima, K. & Izawa, M. (1980) Dev. Cell Biol. 6, 272-284.

175. Ballal, N.R., Goldknopf, I.L., Goldberg, P.A. & Busch, H. (1974) Life Sci. 14, 1835-1845.

176. Smulson, M.E., Stark, P., Gazzoli, M. & Roberts, J. (1975) Exp. Cell Res. 90, 175-182.

177. Whish, W.J.D., Davies, M.I. & Shall, S. (1975) Biochem. Biophys. Res. Comm. 65, 722-730.

178. Smulson, M.E., Schein, P., Gazzoli, M. & Roberts, J. (1975) Exp. Cell Res. 90, 175-182.

179. Shall, S., Goodwin, P., Halldorosson, H., Khan, M.G., Skidmore, C.J. & Tsopanakis, C. (1978) Biochem. Soc. Symp. 42, 103-116.

180. Sudhakar, S., Tew, K.D. & Smulson, M.E. (1979) Cancer Res. 39, 1405-1410.

181. Jacobson, M.K. & Jacobson, E.L. (1978) Fed. Proc. 37, 1347.

182. Sudhakar, S., Tew, K.D., Schein, P.S., Woolley, P.V. & Smulson, M.E. (1979) Cancer Res. 39, 1411-1415.

183. Davies, M.I., Shall, S. & Skidmore, C.J. (1977) Biochem. Soc. Trans. 5, 949-950.

184. Berger, N.A., Sikorski, G.W., Petzold, S.J. & Kurohara, K.K. (1979) J. Clin. Invest. 63, 1164-1171.

185. Miller, E.G. (1977) Fed. Proc. 36, 906.

186. Kidwell, W.R. (1975) J. Biochem. (Tokyo) 77, 6p-7p.

187. Berger, N.A., Sikorski, G.W.. Petzold, S.J. & Kurohara, K.K. (1980) Biochemistry 19, 289-293.

188. Cleaver, J.E. (1969) Proc. Natl. Acad. Sci. USA. 63, 428-435.

189. Setlow, R.B., Regan, J.D., German, J. & Carrier, W.L. (1969) Proc. Natl. Acad. Sci. USA. 64, 1035-1041.

190. Cleaver, J.E. (1973) Cancer Res. 33, 362-369.

191. Berger, N.A., Petzold, S.J. & Berger, S.J. (1979) Biochim. Biophys. Acta 564, 90-104.

192. Nduka, N., Skidmore, C.J. & Shall, S. (1980) Eur. J. Biochem. 105, 525-530.

193. Shall, S., Nduka, N., Omidiji, O. & Zia'ee, A.A. (1980) Dev. Cell Biol. 6, 207-216.

194. Jacobson, M.K. & Narasimhan, G. (1979) Fed. Proc. 38, 619.

195. Durkacz, B.W., Omidiji, O., Gray, D.A. & Shall, S. (1980) Nature (London) 283, 593-596.

196. Lee, Y.C. & Byfield, J.E. (1976) J. Natl. Cancer Inst. 57, 221-224.

197. Kappen, L.S., Goldberg, I.H. & Anantha Samy, T.S. (1979) Biochemistry 18, 5123-5127.

198. Tanuma, S., Enomoto, T. & Yamada, M. (1978) Exp. Cell Res. 117, 421-430.

199. Berger, N.A., Kaichi, A.S., Steward, P.G., Klevecz, R.R., Forrest, G.L. & Gross, S.D. (1978) Exp. Cell Res. 117, 127-135.

200. Mitchison, J.M. (1971) The Biology of the Cell Cycle, Cambridge University Press, 153-156.

201. Roberts, J., Stark, P. & Smulson, M.E. (1973) Biochem. Biophys. Res. Comm. 52, 43-50.

202. Colyer, R.A., Burdette, K.E. & Kidwell, W.R. (1973) Biochem. Biophys. Res. Comm. 53, 960-965.

203. Wielckins, K., Sachsenmaier, W. & Hilz, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 39-43.

204. Caplan, A.I. & Rosenberg, M.J. (1975) Proc. Natl. Acad. Sci. USA. 72, 1852-1857.

205. MacLachlan, J., Bateman, M. & Wolpert, L. (1976) Nature (London) 264, 267-269.

206. Caplan, A.I., Niedergang, C., Okazaki, H. & Mandel, P. (1979) Dev. Biol. 72, 102-109.

207. Burzio, L.O. & Koide, S.S. (1977) Ann. N.Y. Acad. Sci. 286, 398-407.

208. Farzaneh, F. & Pearson, C.K. (1977) Biochem. Soc. Trans. 5, 733-734.

209. Farzaneh, F. & Pearson, C.K. (1979) Dev. Biol. 72, 254-265.

210. Young, R.J. & Sweeney, K. (1978) Biochemistry 17, 1901-1907.

211. Young, R.J. & Sweeney, K. (1979) J. Embryol. Exp. Morph. 49, 139-152.

212. Rastl, E. & Swetly, P. (1978) J. Biol. Chem. 253, 4333-4340.

213. Zlatanova, J.S. & Swetly, P. (1980) Biochem. Biophys. Res. Comm. 92, 1110-1116.

214. Morioka, K., Tanaka, K., Nokuo, T., Ishizawa, M. & Ono, T. (1979) Gann 70, 37-46.

215. Terada, M., Fujiki, H., Marks, P.A. & Sugimura, T. (1979) Proc. Natl. Acad. Sci. USA. 76, 6411-6414.

216. Yamada, M., Shimada, T., Nakayasu, M., Okada, H. & Sugimura, T. (1978) Biochem. Biophys. Res. Comm. 83, 1325-1332.

217. Minaga, T., Morton, L.J., Piper, W.N. & Kun, E. (1978) Eur. J. Biochem. 91, 577-585.

218. Suzuki, Y. & Murachi, T. (1978) J. Biochem. (Tokyo) 84, 977-984.

219. Matinyan, K.S. & Umanski, S.R. (1978) Biokhimya 43, 1196-1205.

220. Umansky, S.R., Matinyan, K.S. & Tokarskaya, V.I. (1980) Eur. J. Biochem. 105, 117-129.

221. Giri, C.P., West, M.H.P. & Smulson, M.E. (1978) Biochemistry 17, 3495-3500.

222. Burzio, L.O., Koide, S.S., Domenech, P.P. & Ruiz-Carrillo, A. (1980) Dev. Cell Biol. 6, 345-356.

223. Giri, C.P., West, M.H.P., Ramirez, M.L. & Smulson, M.E. (1978) Biochemistry 17, 3501-3504.

224. Butt, T.R., Brothers, J.F., Giri, C.P. & Smulson, M.E. (1978) Nuc. Acids Res. 5, 2775-2788.

225. Butt, T.R., Jump, D.B. & Smulson, M.E. (1979) Proc. Natl. Acad. Sci. USA. 76, 1628-1632.

226. Finch, J.T. & Klug, A. (1976) Proc. Natl. Acad. Sci. USA. 73, 1897-1901.

227. Jump, D.B., Butt, T.R. & Smulson, M.E. (1980) Biochemistry 19, 1031-1037.

228. Renz, M., Nehls, P. & Hozier, J. (1977) Proc. Natl. Acad. Sci. USA. 74, 1879-1883.

229. Thoma, F. & Koller, Th. (1977) Cell 12, 101-107.

230. Worcel, A. & Benyajati, C. (1977) Cell 12, 83-100.

231. Byrne, R.H., Stone, P.R. & Kidwell, W.R. (1978) Exp. Cell Res. 115, 277-283.

232. Leiber, U., Kittler, M. & Hilz, H. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1347-1350.

233. Meuth, M., Aufriete, E. & Reichard, P. (1976) Eur. J. Biochem. 71, 39-43.

234. Webb, J.L. (1966) Enzymes and Metabolic Inhibitors, vol. II, Academic Press, N.Y.

235. Wu, J.M., Lennon, M.B. & Suhadolnik, R.J. (1978) Biochim. Biophys. Acta 520, 588-597.

236. Skidmore, C.J., Davies, M.I., Goodwin, P., Halldorsson, H., Lewis, P.J., Shall, S. & Zia'ee, A.A. (1979) Eur. J. Biochem. 101, 135-142.

237. CRC Handbook of Physics and Chemistry, 59th edition.

238. Finan, P.A. & Fothergill, G.A. (1961) J. Chem. Soc. 2824-2825.

239. Bray, H.G., Ryman, B.E. & Thorpe, W.V. (1948) Biochem. J. 43, 561-567.

240. Radziszewski, B. (1885) Ber. Dtsch. Chem. Ges. 18, 355-356.

241. McMaster, L. & Langreck, F.B. (1917) J. Am. Chem. Soc. 39, 103-109.

242. Bogert, M.T. & Beans, H.T. (1904) J. Am. Chem. Soc. 26, 463-499.

243. Cohen, L.A. & Jones, W.M. (1963) J. Am. Chem. Soc. 85, 3402-3406.

244. Reio, L. (1958) J. Chromatog. 1, 338-373.

245. Coulson, C.B. & Evans, W.C. (1958) J. Chromatog. 1, 374-379.

246. Bray, H.G., Thorpe, W.V. & White, K. (1950) Biochem. J. 46, 271-275.

247. Thommes, G.A. & Leininger, E. (1958) Anal. Chem. 30, 1361-1363.

248. Stone, P.R. & Shall, S. (1973) Eur. J. Biochem. 38, 146-152.

249. Yamamoto, H. & Okamoto, H. (1980) Biochem. Biophys. Res. Comm. 95, 474-481.

250. Eisenthal, R. & Cornish-Bowden, A. (1974) Biochem. J. 139, 715-720.

251. Sarma, R.H. & Woronik, C.L. (1972) Biochemistry 11, 170-179.

252. McDaniel, D.H. & Brown, H.C. (1958) J. Org. Chem. 23, 420-427.

253. Nakazawa, K., Ueda, K., Honjo, T., Yoshihara, K., Nishizuka, Y. & Hayaishi, O. (1968) Biochem. Biophys. Res. Comm. 32, 143-149.

254. Guardiola, A.L., Paretsky, D. & McEwen, W. (1958) J. Am. Chem. Soc. 80, 418-421.

255. Adams, G.E. & Dewey, D.L. (1963) Biochem. Biophys. Res. Comm. 12, 473-477.

256. Adams, G.E. & Cooke, M.S. (1969) Int. J. Rad. Biol. 15, 457-471.

257. Webb, J.L. (1966) Enzymes and Metabolic Inhibitors, vol. III, 421-585.

258. Abakumov, G.Z. & Lukienko, P.I. (1979) Bull. Exp. B. Med. 86, 1046-1047.

259. Moore, G.E., Sandberg, A.A. & Ulrich, K. (1966) J. Natl. Canc. Inst. 36, 405-421.

260. Takakusu, A., Lazarus, H., Levine, H., McCoy, T.A. & Foley, G.E. (1968) Exp. Cell. Res. 49, 226-

261. Berger, N.A. (1978) Methods in Cell Biology 20, 325-340.

262. Nisselbaum, J.S. & Green, S. (1969) Anal. Biochem. 27, 212-217.

263. Goodwin, P.M., Lewis, P.J., Davies, M.I., Skidmore, C.J. & Shall, S. (1978) Biochim. Biophys. Acta 543, 576-582.

264. Jacobson, K.B. & Kaplan, N.O. (1957) J. Biophys. Biochem. Cytol. 3, 31.

265. Fukushima, M., Okayama, H., Takahasi, Y. & Hayaishi, O. (1976) J. Biochem. 80, 167-176.

266. Tamulevicius, P., Streffer, C., Roscic, O. & Hubert, E. (1979) Biochem. J. 178, 467-473.

267. Mellors, A., Lun, A.K.L. & Peled, O.N. (1975)
Can. J. Biochem. 53, 143-148.

268. Muller, H. & Schuber, F. (1980) Eur. J. Biochem. 104,
489-500.

269. Bock, K.W., Gang, V., Beer, H.P., Kronau, R. &
Grunicke, H. (1968) Eur. J. Biochem. 4, 357-363.

270. Pekala, P.H. & Anderson, B.M. (1978)
J. Biol. Chem. 253, 7453-7459.

271. Olivera, B.M., Reichsteiner, M.C., Ramos, P.M.,
Cruz, L.J. & Hillyard, D. (1980) Dev. Cell Biol. 6, 85-98.

272. Artman, M. & Seeley, R.J. (1979) Arch. Biochem. Biophys.
195, 121-127.

273. Artman, M. & Seeley, R.J. (1978) Science 202, 1293-1295.

274. Moss, J., Stanley, S.J. & Oppenheimer, N.J. (1979)
J. Biol. Chem. 254, 8891-8894.

275. Moss, J., Stanley, S.J. & Watkins, P.A. (1980)
J. Biol. Chem. 255, 5838-5840.

276. Dickerman, H.W., San Pietro, A. & Kaplan, N.O. (1962)
Biochim. Biophys. Acta 62, 230-244.

277. Chem. Abs. 92 210772.

278. Webb, J.L. (1966) Enzymes and Metabolic Inhibitors,
vol. II, 488.

279. Dietrich, L.S. & Muniz, O. (1972)
Biochemistry 11, 1691-1695.

280. Dietrich, L.S. (1971)
Meth. Enzymol. 18B, 144-149.

281. Blake, R.L. & Kun, E. (1971)
Meth. Enzymol. 18B, 113-123.

282. Bray, H.G., Lake, H.J., Neale, F.C., Thorpe, W.V. &
Wood, P.B. (1948) Biochem. J. 42, 434-443.

283. Bray, H.G., Henry, H. & Stacey, M. (1946)
Biochem. J. 40, 134-139.

284. Bray, H.G., Thorpe, W.V. & Wood, P.B. (1949)
Biochem. J. 44, 39-45.

285. Bray, H.G., James, S.P., Ryman, B.E. & Thorpe, W.V. (1948) Biochem. J. 42, 274-281.

286. Kidwell, W.R., Stone, P.R. & Johnson, G.S. (1980) Dev. Cell. Biol. 6, 73-84.

287. Kidwell, W.R. & Burdette, K.E. (1974) Biochem. Biophys. Res. Comm. 61, 766-773.

288. Nduka, N. & Shall, S. (1980) Biochem. Biophys. Res. Comm. 96, 997-1002.

289. Webb, J.L. (1966) Enzymes and Metabolic Inhibitors, vol. II, 372.

290. Davies, R.C., Auld, D.S. & Vallee, B.L. (1968) Biochem. Biophys. Res. Comm. 31, 628-633.

291. Bredehorst, R., Lengyel, H., Hilz, H., Staerk, D. & Siebert, G. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 559-562.

292. Dfordjevic, B., Evans, R.G., Perz, A.G. & Wiell, M.K. (1969) Nature 224, 803-804.

293. Painter, R.B. & Young, B.R. (1972) Mut. Res. 14, 225-235.

294. Gautschi, J.R., Young, B.R. & Painter, R.B. (1972) Biochim. Biophys. Acta 281, 324-328.

295. Smith, C.A. & Hanawalt, P.C. (1976) Biochim. Biophys. Acta 447, 121-132.

296. Strayer, D.R. & Boyer, P.D. (1978) J. Mol. Biol. 120, 281-295.

297. Hilton, J. & Walker, M.D. (1977) Biochem. Biophys. Res. Comm. 77, 909-914.

298. Ohtsuka, E., Tanigawa, Y. & Koide, S.S. (1975) Experientia 31, 175-177.

299. Yamada, M., Nagao, M., Miwa, M. & Sugimura, T. (1974) Biochem. Biophys. Res. Comm. 56, 1093-1099.

300. Utakoji, T., Hosoda, K., Umezawa, K., Sawamura, M., Matsushima, T., Miwa, M., & Sugimura, T. (1979) Biochem. Biophys. Res. Comm. 90, 1147-1152.

301. Babu, K.A., Shah, V.C. & Lakhotia, S.C. (1980) Ind. J. Exp. Biol. 18, 329-332.

302. Rochette-Egly, C., Ittel, M.E., Bilen, J. & Mandel, P. (1980) FEBS Lett. 120, 7-11.

303. Reuben, R.C., Khanna, P.L., Gazitt, Y., Breslow, R., Rifkind, R.A. & Marks, P.A. (1978) J. Biol. Chem. 253, 4214-4218.

304. Farzenah, F., Shall, S. & Zalin, R. (1980) Dev. Cell Biol. 6, 217-226.

305. Yu, F. & Feigelson, P. (1972) Proc. Natl. Acad. Sci. USA. 69, 2833-2837.

306. Kitamura, A., Tanigawa, Y., Doi, S., Kawakami, K. & Shimoyama, M. (1980) Arch. Biochem. Biophys. 204, 455-463.

307. Todhunter, J.A., Weissbach, H. & Brot, N. (1978) J. Biol. Chem. 253, 4514-4516.

SECTION 7

APPENDIX

SEPARATION OF THIOL-CONTAINING PROTEINS AND ANALYSIS OF ADP-RIBOSYLATION

(performed in co-operation with Dr P. R. Stone)

FRACTIONATION OF IN VITRO ADP-RIBOSYLATED NUCLEAR PROTEINS USING PYRIDYL DISULPHIDE SEPHAROSE CHROMATOGRAPHY

Michael R. Purnell, Peter R. Stone and William J.D. Whish,
Department of Biochemistry, School of Biological Sciences,
University of Bath, Claverton Down, Bath BA2 7AY, England.

ABSTRACT

Pig thymus nuclei and 0.5M NaCl extracts of these nuclei were incubated with [^{3}H]NAD and the protein subsequently fractionated by covalent thiol-disulphide exchange chromatography. The extent of ADP-ribosylation of such fractionated thiol and non-thiol containing proteins was studied using SDS-polyacrylamide gel electrophoresis. Differences in the ADP-ribosylation of these two families of proteins were seen. Furthermore, differences in the hydroxylamine sensitivities of the ADP-ribose protein linkages were observed when analysed using a filter disc assay. This finding was confirmed using gel electrophoresis.

Running Title: Fractionation of ADP-ribose proteins.

Subject Category: Chromatographic and Electrophoretic Techniques.

FOOTNOTES

1. Abbreviations used: ADP-ribose, adenosine diphosphate ribose; HMG proteins, high mobility group proteins; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid.

In order to determine the biological significance of poly ADP-ribose[1] synthesis and ADP-ribosylation of nuclear proteins (for a recent review see (1)) it is clearly necessary to identify and characterise those proteins which are ADP-ribosylated. To date several methods have been used to resolve the possible acceptor proteins including CsCl-equilibrium density gradient centrifugation followed by hydroxyapatite chromatography and two-dimensional gel electrophoresis (2) and separation of ADP-ribosylated proteins from non-modified proteins by affinity chromatography on boronate columns (3-5). Isolation and analysis of specific classes of nuclear proteins eg, histones and the high mobility group non-histone proteins (HMG) has been the most commonly used approach because of the relative ease with which such proteins can be purified (1,6,7). However, such methodology may not be completely satisfactory since it has recently been shown that the chromatographic and electrophoretic properties of the histones and non-histones are drastically altered when such proteins are ADP-ribosylated (5). Indeed, most of the ADP-ribosylated histones synthesised in vitro in Ehrlich ascites tumour cells appeared in the non-histone protein fraction when separated using Bio-Rex 70 chromatography (5). In order to avoid such possible complications we have employed a different methodology, which is not influenced by ADP-ribosylation of proteins, to fractionate nuclear proteins. Using covalent chromatography by thiol-disulphide exchange (8) we have fractionated nuclear proteins into thiol and non-thiol containing fractions. It is this methodology and the preliminary analysis of the ADP-ribosylation of these two protein fractions which we wish to present in this report.

MATERIALS AND METHODS

[Adenosine-^{3}H]NAD (1mCi/ml, 20mCi/μmoles) was synthesised from [^{3}H]ATP (Radiochemical Centre, Amersham) by the method of Ohtsu and Nishizuka (9). Sepharose-4B was obtained from Pharmacia, epichlorohydrin from Fisons

Laboratories and 2,2'-dipyridyl disulphide from Sigma Chemical Company.

Isolation of Pig Thymus Nuclei: The isolation was performed as described by Khan and Shall (10).

Preparation of 0.5M NaCl Extract: The extract was prepared as previously described (11).

Preparation of Pyridyl Disulphide Sepharose: Sepharose-4B was activated with epichlorohydrin and the pyridyl disulphide derivative synthesised using 2,2'-dipridyl disulphide as described by Axen et al (12).

Incorporation of [^{3}H]NAD: Samples (200µl of nuclei or 0.5M NaCl extract) were incubated at 26°C for 5 minutes with 50µl [^{3}H]NAD in a total volume of 1ml which contained 1mM dithiothreitol, 10mM $MgCl_2$, 50mM triethanolamine/HCl pH 8.2. Then 4ml "urea buffer" (5M urea, 2M KCl, 1mM EDTA, 50mM sodium acetate pH 6) were added and the sample mixed thoroughly. For larger scale incubations the samples, following incubation, were precipitated with 20% TCA($^w/_v$) (final concentration) and centrifuged and washed with ethanol prior to solubilisation in "urea buffer".

Pyridyl Disulphide Sepharose Chromatography: 3ml of the sample were mixed with 3ml (packed volume) of the sepharose which had been equilibrated in "urea buffer". The mixture was left for at least 30 minutes at room temperature and mixed occasionally. The sample was then poured into a small column (1cm, diameter) and washed with 5ml volume of urea buffer. 5ml fractions were collected and 50µl aliquots taken for determination of radioactivity. When no more radioactivity eluted 2.5ml urea buffer containing 0.5M 2-mercaptoethanol were applied to the column which was left for 40 minutes at room temperature. The column was then eluted with 2.5ml volumes of urea buffer - 0.5M 2-mercaptoethanol and 2.5ml fractions collected until all the radioactivity had eluted. The eluted fractions were analysed for total radioactivity by counting 50µl aliquots in 0.5% PPO-70% toluene-30% triton X-100, for acid precipitatable radioactivity

using the filter disc assay as previously described except that all TCA washes were 20% ($^{w}/_{v}$) TCA (11) and for protein content using the Coomassie Blue G-250 method of Sedmak and Grossberg (13).

Analysis of Pyridyl Disulphide Sepharose Fractions: The pooled fractions from the column were precipitated with 25% TCA (final concentration) and left on ice for 4 hours before centrifuging (20 minutes, 10,000xg) and washing twice with 20% TCA and twice with ethanol. Aliquots of the unfractionated samples prior to the column chromatography were also treated in the same way. Aliquots of the precipitated washed protein samples (10-50μg) treated $\pm$0.4M NH_2OH pH 7 for 30 minutes at 37^{o}C were analysed on SDS polyacrylamide gels essentially as described by Weber and Osborn (14) except that the gels were 7.5% acrylamide, the pH of the phosphate buffer was 6.3 and 5M urea was included in all solutions. The gels were sliced and the radioactivity determined using the one-step procedure of Aloyo (15).

RESULTS AND DISCUSSION

As far as we are aware, covalent thiol-disulphide exchange column chromatography (8) has not been applied to the analysis of total nuclear proteins and in particular to ADP-ribosylated nuclear proteins. Such a method has many advantages amongst which are the ability to perform separations under conditions which favour the stability of ADP-ribose-protein conjugates ie, low pH and denaturing conditions. Furthermore, unlike other protein separation techniques routinely employed, the modification of proteins by ADP-ribosylation should have no effect on the fractionation. We have applied such a chromatographic procedure to an analysis of ADP-ribosylated nuclear proteins from pig thymus.

After using the standard incubation conditions for ADP-ribosylation of nuclear proteins as outlined in Materials and Methods it was observed that a proportion of the acid-insoluble radioactivity remained bound to 2-pyridyl disulphide Sepharose and which could only be eluted with 2-mercaptoethanol

or dithiothreitol. The kinetics of such binding are shown in Fig. 1 and it can be seen that binding is complete in 20 minutes. This material represents thiol protein-bound [^{3}H]mono or oligo (ADP-ribose) because in control experiments in which nuclei were incubated with [^{3}H]NAD in the presence of 3-aminobenzamide, an extremely potent inhibitor of poly(ADP-ribose) synthetase (11), no radioactivity remained bound to the column (data not shown). Incubation of pig thymus nuclei with [^{3}H]NAD followed by solubilisation and covalent chromatography gives an elution profile as seen in Fig. 2. The protein content (Fig. 2A) indicates that approximately 14% of the total nuclear protein contains free thiol groups. The same distribution of protein between the thiol and non-thiol protein fractions, which represents a quantitative recovery of total protein applied, was also seen when proteins from nuclei incubated in the absence of any [^{3}H]NAD were chromatographed (data not shown). Analysis of the total radioactivity in the eluted fractions (Fig. 2B) reveals that the majority of the label is not retained and represents [^{3}H]NAD and [^{3}H]ADP-ribosylated proteins which do not contain a free thiol. However, a peak of radioactivity is retained on the column which elutes with 0.5M 2-mercaptoethanol and represents [^{3}H]ADP-ribosylated thiol containing proteins. The extent of ADP-ribosylation of both the thiol and non-thiol proteins was determined by analysis of the acid-insoluble radioactivity (ie, protein bound ADP-ribose) in each of the fractions (Fig. 2C). It is seen that approximately 30% of the [^{3}H]ADP-ribose incorporated in nuclei is attached to proteins which contain a thiol group and thus retained on the column. The results obtained using isolated nuclei are summarised in Table 1. It is noteworthy that the extent of ADP-ribosylation is greater in the thiol containing proteins as evidenced by a 2.5 fold higher specific activity of protein bound [^{3}H]ADP-ribose in this fraction. The specific activity of the two fractions of proteins isolated from a 0.5M NaCl nuclear extract which had been incubated with [^{3}H]ADP-ribose in this fraction.

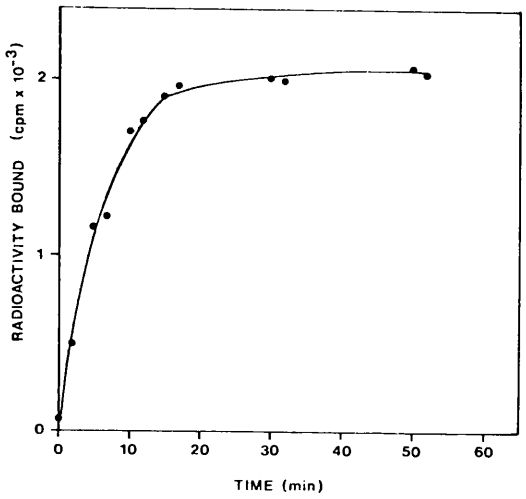


Fig. 1 Kinetics of binding of [^{3}H]ADP-ribosylated thiol proteins to pyridyl disulphide Sepharose. 2ml of a solubilised 0.5M NaCl nuclear extract which had been incubated with [^{3}H]NAD and processed as in Materials and Methods was mixed with 2ml packed pyridyl disulphide Sepharose. At various times 200µl aliquots were taken and filtered on a GF/C glass fibre filter which was then placed in 3ml urea buffer - 0.5M 2-mercaptoethanol and left for 45 minutes at room temperature before centrifuging and counting 0.5ml aliquots of the supernatant for radioactivity.

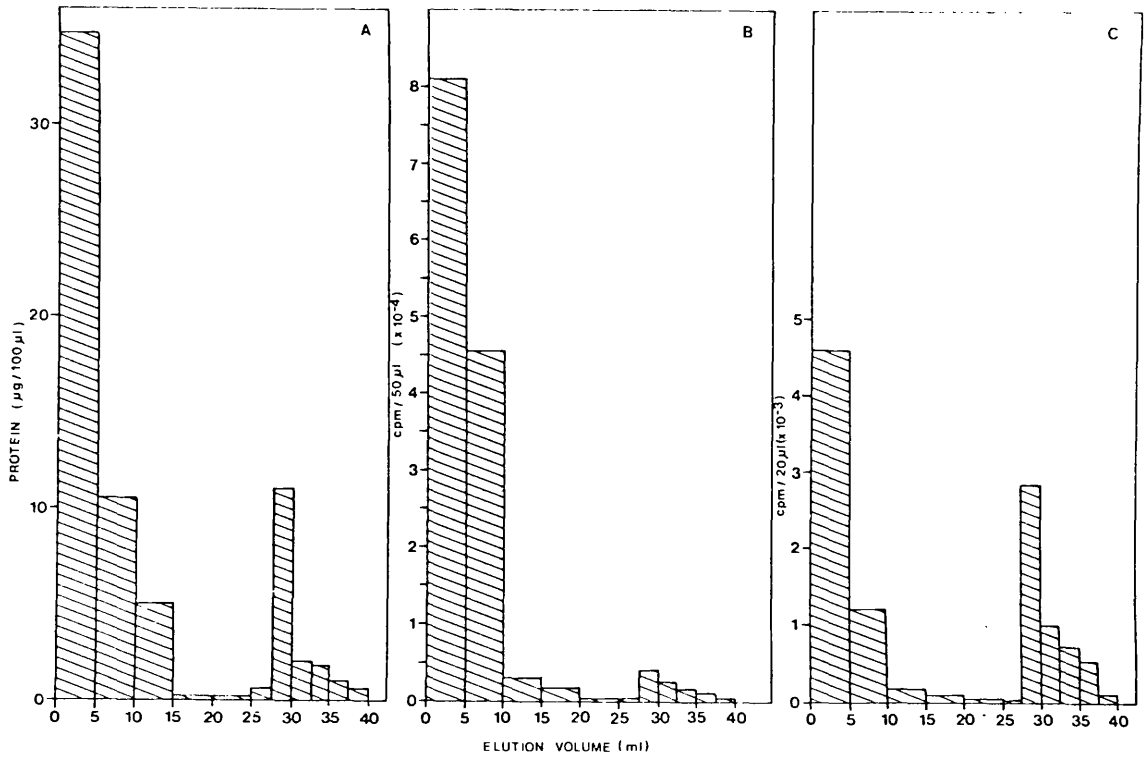


Fig. 2 Elution profile of nuclear proteins on pyridyl disulphide Sepharose. 3ml of a solubilised nuclear preparation which had previously been incubated with [^{3}H]NAD was chromatographed as described in Materials and Methods. 5 x 5ml fractions were collected before eluting with 2.5ml volumes of urea buffer - 0.5M 2-mercaptoethanol. A: Protein, B: Total radioactivity, C: Acod-insoluble radioactivity.

The specific activity of the two fractions of proteins isolated from 0.5M NaCl nuclear extract which had been incubated with [^{3}H]NAD is more than 20-fold greater than the corresponding fractions from nuclei. Such an increase in specific activity may arise from either increased ADP-ribosylation in the 0.5M NaCl extract or an enrichment of the acceptor proteins in this extract. We are currently investigating these possibilities.

It has been established that mono and poly ADP-ribose is covalently attached to proteins via two linkages only one of which is hydrolysed by neutral hydroxylamine. Thus, we have analysed the lability of the ADP-ribose protein conjugates in each of the protein fractions towards hydroxylamine and NaOH, which quantitatively hydrolyses all ADP-ribose protein conjugates (16). Table 2 summarises the results obtained for all the fractions. For proteins fractionated from nuclei, the sensitivity towards NaOH is equal in both fractions, whereas hydroxylamine sensitivity of the conjugates from the thiol proteins is 2-fold greater than in the non-thiol proteins. These latter proteins also show greater lability at pH 6. The hydroxylamine and pH 6 sensitivities of the total unfractionated ADP-ribosylated proteins, isolated from [^{3}H]NAD incubated 0.5M NaCl nuclear extract, are both greater than in the corresponding proteins from nuclei. As seen in Table 2 this difference is due to an increased sensitivity of the ADP-ribose conjugates in the non-thiol containing proteins. HMG proteins are known acceptors of ADP-ribose (see 1) and in view of their properties should represent a higher proportion of the protein in the non-thiol containing fraction from the 0.5M NaCl nuclear extract compared with whole nuclei (17). We have recently observed that the linkage between ADP-ribose and HMG proteins 1 and 2 is very labile even at neutral pH (Stone and Whish unpublished data) and this could well explain the higher sensitivity in the non-thiol fraction.

Protein fractions have also been analysed by SDS polyacrylamide gel electrophoresis following incubation in the presence and absence of hydroxyl-

amine. As might be expected the stained protein profiles on the gels are complex with very many protein bands being visible (data not shown). In view of the recent observation which shows that the electrophoretic properties of proteins are drastically altered when ADP-ribosylated, analysis of protein profiles is of little value for the identification of ADP-ribosylated species. For this reason only the [^{3}H]ADP-ribose profiles are presented (Figs. 3 and 4). The distribution of radioactivity obtained when total nuclear proteins are analysed is shown in Fig. 3A. The isotope is distributed throughout the gel with a major peak at slice number 5. When total nuclear proteins were analysed after treatment with hydroxylamine a reduction in the radioactivity electrophoresing at this position is observed (Fig. 3D). This clearly indicates a partial resistance of the ADP-ribose conjugate(s) to hydroxylamine. Such a result could arise from either the presence of a single protein ADP-ribosylated through two different linkages or several proteins being present which possess different sensitivities. The latter possibility is favoured because this major peak is seen in both the non-thiol (Fig. 3B) and the thiol proteins (Fig. 3C). The clear separation of the thiol containing histone H3 and the rest of the histones by thiol-disulphide exchange chromatography (Figs. 3A-C) excludes the possibility that this peak results from cross-contamination between the two protein fractions. Furthermore, because this peak in the two fractions exhibits different hydroxylamine sensitivities (Fig. 3E and 3F) it is most unlikely that this peak is a single protein. It is also noteworthy that the faster migrating proteins are more sensitive to hydroxylamine than those proteins which migrate slowly (Fig. 3D-F). Clear differences in the radioactive profile are seen between the thiol (Fig. 3C) and the non-thiol (Fig. 3B) proteins as well as in their sensitivity to hydroxylamine (Fig. 3E and 3F). Results obtained from a 0.5M NaCl nuclear extract (Fig. 4) also show differences in the radioactive profiles (Fig. 4A-C) as well as in their hydroxylamine sensitivities (Fig. 4D-F). These sensitivities are clearly greater than in

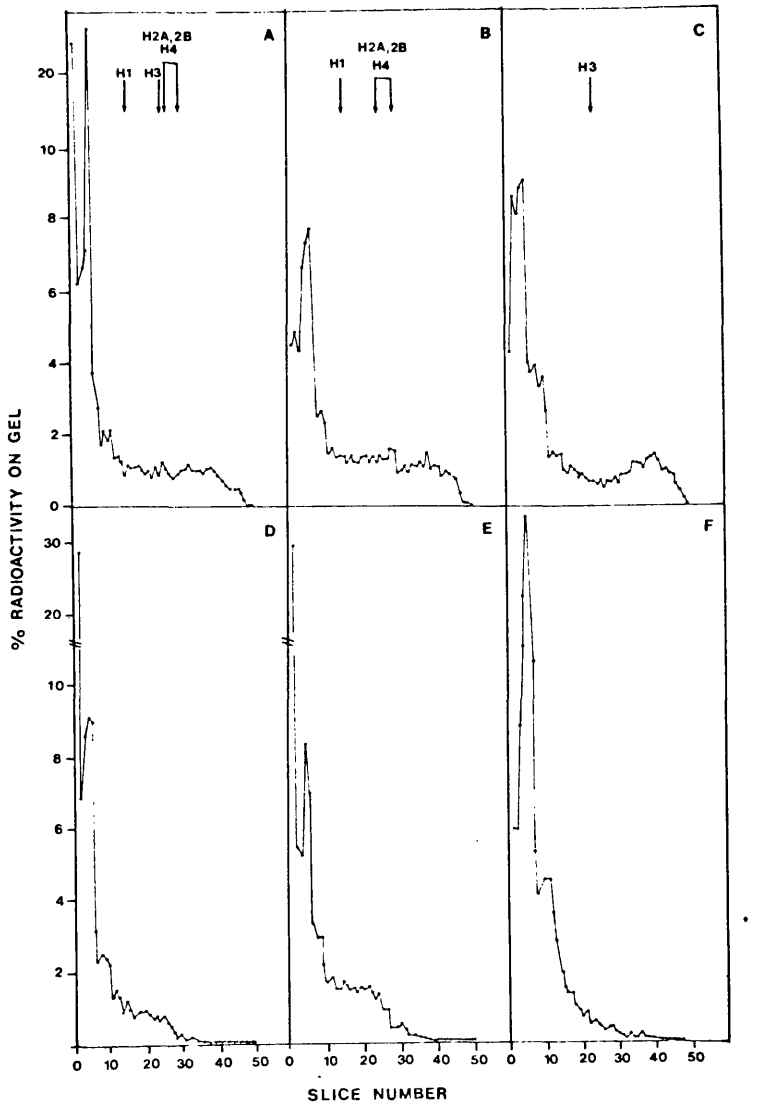


Fig. 3 SDS polyacrylamide gel electrophoresis of nuclear, [^{3}H]ADP-ribosylated proteins. Samples were processed as described in Methods. A: Total protein, B: Non-thiol protein, C: Thiol protein, D: Total protein + hydroxylamine, E: Non-thiol protein + hydroxylamine, F: Thiol protein + hydroxylamine. The arrows mark the position of the histones in the samples.

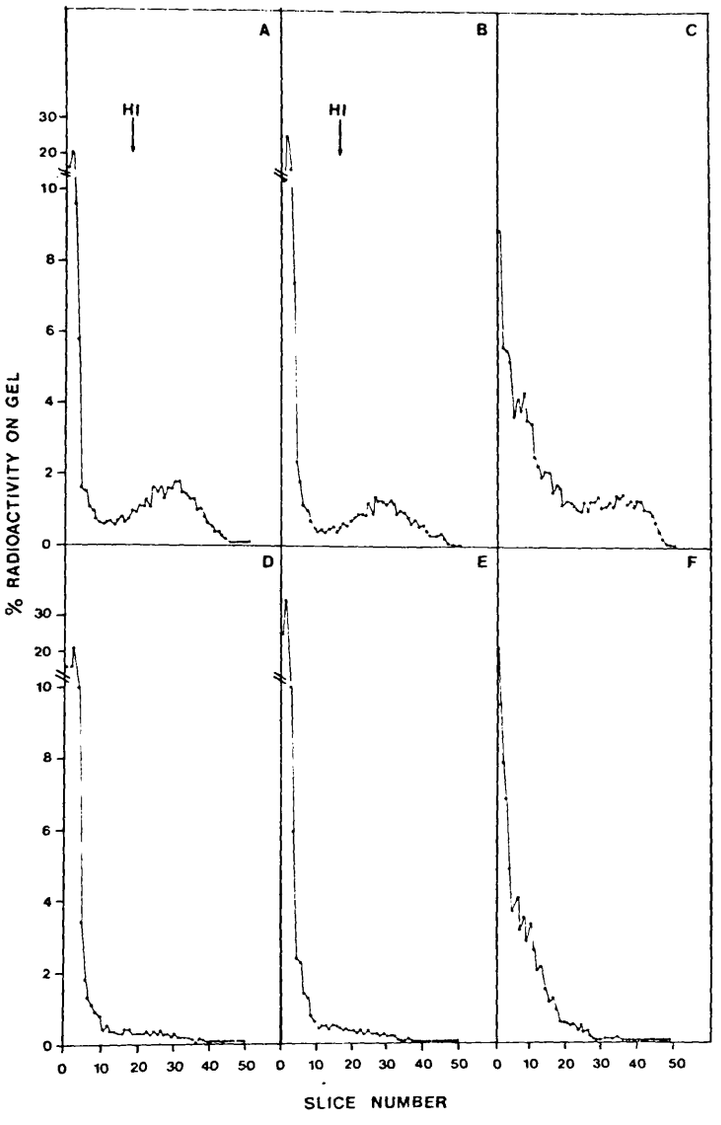


Fig. 4 SDS polyacrylamide gel electrophoresis of 0.5M NaCl nuclear extract [^{3}H]ADP-ribosylated proteins. Legend is exactly same as for Fig. 3.

the case of nuclear proteins (Fig. 3D-3F) which is consistent with the results shown in Table 2. A comparison of the profiles obtained with nuclei and the 0.5M NaCl extract shows marked differences in their composition. A further point is the absence, in the 0.5M NaCl extract, of the major peak at slice 5 (Fig. 4) as well as an absence of the majority of the hydroxylamine resistant material present in the non-thiol proteins from nuclear (Figs. 3E and 4E).

The methodology presented in this report clearly facilitates the separation of nuclear thiol and non-thiol proteins. Analysis of the ADP-ribosylation of these two fractions indicates marked differences both in the composition of ADP-ribose conjugates and in their properties. Furthermore, such separation seems to result in a partial enrichment of the acceptor proteins in the thiol fraction as evidenced by the increased specific activity of the incorporated ADP-ribose in this fraction (Table 1). As an extension of this work the non-thiol containing proteins could be further fractionated by reduction of any disulphide bonds present in this fraction and subsequent rechromatography resulting in two fractions each of which will be less complex in their protein composition.

Such thiol-disulphide exchange chromatography provides an excellent step in the isolation and purification of specific ADP-ribose acceptor proteins. An obvious candidate for such analysis is poly(ADP-ribose) synthetase which has been shown to be capable of self ADP-ribosylation (18) and, moreover, to contain thiol groups (19,20). Preliminary results from this laboratory indicate that the synthetase may be isolated using this technique. An exciting application of this methodology is to analyse those proteins which are ADP-ribosylated *in vivo*. We are currently proceeding in this direction.

ACKNOWLEDGEMENTS

We thank the Science Research Council (Support to MRP, PRS and WJDW), the Medical Research Council (Support to WJDW) and Miles Laboratories (Support to WJDW and MRP).

REFERENCES

1. Purnell, M.R., Stone, P.R. and Whish, W.J.D. (1980) Biochem. Soc. Trans. 8, 215-227.

2. Rickwood, D., MacGillivray, A.J. and Whish, W.J.D. (1977) Eur. J. Biochem. 79, 589-598.

3. Okayama, H., Ueda, K. and Hayaishi, O. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1111-1115.

4. Minaga, T., Romaschin, A.D., Kirsten, E. and Kun, E. (1979) J. Biol. Chem. 254, 9663-9668.

5. Adamietz, P., Klapproth, K. and Hilz, H. (1979) Biochem. Biophys. Res. Commun. 91, 1232-1238.

6. Hilz, H. and Stone, P.R. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1-58.

7. Hayaishi, O. and Ueda, K. (1977) Annual Rev. Biochem. 46, 95-116.

8. Brocklehurst, K., Carlsson, J., Kierstan, M.P.J. and Crook, E.M. (1974) Methods Enzymol. 34B, 531-544.

9. Ohtsu, E. and Nishizuka, Y. (1971) Methods Enzymol. 18B, 127-131.

10. Khan, M.G. and Shall, S. (1976) Biochem. Soc. Trans. 4, 778-781.

11. Purnell, M.R. and Whish, W.J.D. (1980) Biochem. J. 185, 775-777.

12. Axén, R., Drevin, H. and Carlsson, J. (1975) Acta Chemica Scandinavica B, 29, 471-474.

13. Sedmak, J.J. and Grossberg, S.E. (1977) Anal. Biochem. 79, 544-552.

14. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.

15. Aloyo, U.J. (1979) Anal. Biochem. 99, 161-164.

16. Adamietz, P. and Hilz, H. (1976) Hoppe Seyler's Z. Phys. Chem. 357, 527-534.

17. Goodwin, G. and Johns, E.W. (1977) Methods Cell Biol. XVI, 257-267.

18. Yoshihara, K., Hashida, T., Yoshihara, H., Tanaka, Y. and Ohgushi, H. (1977) Biochem. Biophys. Res. Commun. 78, 1281-1288.

19. Ito, S., Shizuta, Y. and Hayaishi, O. (1979) J. Biol. Chem. 254, 3647-3651.

20. Holtlund, J., Kristensen, T. and Sletten, K. (1980) Biochem. J. 185, 779-782.

TABLE 1

Distribution of protein and radioactivity

on pyridyl disulphide Sepharose columns

Sample	Fraction	Acid Insoluble Radioactivity (cpm)	Protein (μg)	cpm/μg protein
	Total	2.15×10^6 (100%)	2930 (100%)	734
Nuclei	Non-Thiol	1.5×10^6 (70%)	2500 (85.6%)	600
	Thiol	0.65×10^6 (30%)	430 (14.4%)	1,511
	Total	6.35×10^6 (100%)	266.9 (100%)	23,782
0.5M NaCl	Non-Thiol	4.1×10^6 (64.6%)	190 (71.2%)	21,578
Extract	Thiol	2.25×10^6 (35.4%)	76.9 (28.8%)	29,258

Samples were incubated and processed as described in Methods.

TABLE 2

The effect of NaOH and neutral hydroxylamine on the stability of the ADP-ribosylated protein fractions

Sample	Treatment	% Acid-Insoluble Radioactivity Released	
		Nuclei	0.5M NaCl Extract
	Control	5.2	23.5
Total	$+NH_2OH$	15.5	31.4
Protein	+NaOH	55.2	50.0
	Control	0	24.2
Non-Thiol	$+NH_2OH$	11.9	30.3
Protein	+NaOH	58.7	37.9
	Control	15.3	14.5
Thiol	$+NH_2OH$	24.0	26.8
Protein	+NaOH	56.4	57.3

The TCA precipitated and washed protein fractions were dissolved in 2ml urea buffer (pH 6) and 100µl aliquots incubated for 30 minutes at 37°C with 100µl H_2O (control), 100µl 0.8M NH_2OH pH 7 or with 100µl 0.2N NaOH. 20µl aliquots were taken at time 0 and 30 minutes and processed for acid-insoluble radioactivity using the filter disc assay.

SECTION 8

PUBLICATIONS

PUBLICATIONS

1. Purnell, M.R. & Whish, W.J.D. (1977) 11th FEBS Meeting Abstract No. A2-3-164-2.

2. Purnell, M.R. & Whish, W.J.D. (1980) Biochem. J. 185, 775-777.

3. Purnell, M.R. & Whish, W.J.D. (1980) Biochem. Soc. Trans. 8, 175-176.

4. Purnell, M.R., Stone, P.R. & Whish, W.J.D. (1980) Biochem. Soc. Trans. 8, 215-227.

5. Purnell, M.R., Stone, P.R., Surowy, C.J. & Whish, W.J.D. (1980) Dev. Cell Biol. 6, 285-294.

6. Purnell, M.R., Stone, P.R., Surowy, C.J. & Whish, W.J.D. (1981) Biochem. Soc. Trans. 9(1) in the press.

7. Stone, P.R., Purnell, M.R. & Whish, W.J.D. (1980) Anal. Biochem. 109 in the press.

Biochem. J. (1980) 185, 775–777
Printed in Great Britain

Novel Inhibitors of Poly(ADP-Ribose) Synthetase

Michael R. PURNELL and William J. D. WHISH
Department of Biochemistry, University of Bath, Claverton Down, Bath, Avon BA2 7AY, U.K.

(*Received 8 November 1979*)

In a search for new inhibitors of the nuclear enzyme poly(ADP-ribose) synthetase, it was found that various benzamides substituted in the 3-position were the most inhibitory compounds found to date. Two of the benzamides, 3-aminobenzamide and 3-methoxybenzamide, were found to be competitive inhibitors, with K_i values of less than 2 μM.

Despite a large amount of research, the function fulfilled by the modification of nuclear proteins by mono- and poly-(ADP-ribose) remains unknown (Hilz & Stone, 1976; Hayaishi & Ueda, 1977; Purnell *et al.*, 1980). Workers have suggested putative roles in the regulation of DNA synthesis, transcription and repair, cell-cycle events and cellular differentiation and development (for a summary, see Purnell *et al.*, 1980). One approach to determine the function is to inhibit the enzyme responsible for ADP-ribosylation, poly(ADP-ribose) synthetase (EC 2.4.99.–), *in vivo* and look for cellular dysfunction. Various compounds are known to inhibit poly(ADP-ribose) synthetase *in vitro*. Unfortunately, the results obtained from the study of cells treated with any of the inhibitors used to date are of limited value, because these compounds lack physiological specificity. Nicotinamides (Clark *et al.*, 1971) affect the synthesis of NAD and may deplete cellular phosphoribosyl diphosphate pools (Leiber *et al.*, 1973), resulting in a decrease in nucleotide synthesis. Thymidine (Preiss *et al.*, 1971) is known to inhibit DNA synthesis by depleting dCTP concentrations in the cell (Meuth *et al.*, 1976). Methylated xanthines and cytokinins, also inhibitors of poly(ADP-ribose) synthetase, are known to affect cyclic phosphodiesterase (EC 3.1.4.17) (Levi *et al.*, 1978). Thus any alteration of cellular processes observed on treatment of cells with the above compounds cannot be ascribed directly to inhibition of poly(ADP-ribose) synthetase.

In this laboratory, a search has been made for physiologically specific inhibitors of the enzyme that can enter the cell and affect only poly(ADP-ribose) synthetase. Presented below are the results obtained from the study of various benzamides and structurally related compounds on poly(ADP-ribose) synthetase activity *in vitro*.

Materials and Methods

Nicotinamide, nicotinic acid and 2- and 4-aminobenzoic acids were obtained from BDH Ltd., Poole, Dorset, U.K. 6-Aminonicotinamide, thymidine and NAD^+ were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 3-Nitrobenzoyl chloride, 3-acetylpyridine, 3-nitrobenzamide, 3-nitroacetophenone, 3-cyanophenol, 3-hydroxybenzoic acid and 3-methoxybenzamide were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 3-Bromobenzoyl chloride was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

3-Aminobenzamide was synthesized from 3-nitrobenzamide by catalytic hydrogenation, and 3-amino-*N*-methylbenzamide by hydrogenation of 3-nitrobenzamidomethane (made from 3-nitrobenzoyl chloride and methylamine). 3-Acetamidobenzamide and 3-succinylaminobenzamide [3-(3-carboxypropionyl)aminobenzamide] were prepared from 3-aminobenzamide and the corresponding acid anhydride. 3-Bromobenzamide was prepared from the acid chloride and ammonium acetate in acetone (Finan & Fothergill, 1961), and 3-hydroxybenzamide from 3-cyanophenol by treatment with slightly alkaline H_2O_2 by the method of Radziszewski (1885).

Isolation of nuclei and extraction of poly(ADP-ribose) synthetase

Nuclei were isolated from thymus of a freshly slaughtered pig as described by Khan & Shall (1976). The nuclei were resuspended in 0.5 M-NaCl/100 mM-triethanolamine (adjusted to pH 8.2 with conc. HCl/10 mM-$MgCl_2$/2 mM-dithiothreitol. After 10 min on ice, the suspension was centrifuged for 60 min at 100000 *g*. The supernatant, containing the enzyme, was immediately frozen and used for subsequent enzyme assays.

0306-3275/80/030775-03 $1.50/1

Poly(ADP-ribose) synthesis was measured by the incorporation of [*adenine*-^{3}H]NAD$^+$ (synthesized from [^{3}H]ATP; The Radiochemical Centre) into acid-insoluble material. The assay mixture consisted of 100mM-triethanolamine/HCl, pH8.2, 10mM-$MgCl_2$, 2mM-dithiothreitol and 50μM-[^{3}H]NAD$^+$ (2μCi/nmol), in 180μl. The reaction was started by the addition of 20μl of the 0.5M-NaCl extract of nuclei. After 5min at 26°C, a 20μl portion was applied to a filter disc (Whatman grade 1; 2.0cm diam.), which had been presoaked in 20% (w/v) trichloroacetic acid in diethyl ether and dried at room temperature. The disc was left in aq. 20% trichloroacetic acid for 30min on ice, washed four times with 1% trichloroacetic acid, once with ethanol and once with diethyl ether. After the disc had dried at room temperature, the radioactivity was determined by counting in 0.5% (w/v) 2,5-diphenyloxazole in toluene, by using a Packard liquid-scintillation counter.

Protein was determined by the method of Sedmak & Grossberg (1977), with bovine serum albumin as standard.

Results and Discussion

Benzamide, a close analogue of nicotinamide, was first shown to be an inhibitor of poly(ADP-ribose) synthetase by Shall (1975). It has the major advantage over other analogues of nicotinamide in that, since it lacks the ring nitrogen of nicotinamide, it cannot be metabolized by NAD-biosynthetic enzymes. The major drawbacks to its use as an inhibitor for physiological studies are its extremely low solubility and very hydrophobic nature. During the course of this work, we have examined several benzamides substituted in the 3-position for their ability to inhibit the enzyme. Table 1 shows the effect of these compounds at 50μM (equimolar with substrate) on the activity of poly(ADP-ribose) synthetase activity *in vitro*. All the benzamides were more potent inhibitors than either nicotinamide or thymidine in this system. With the exception of 3-nitrobenzamide, inhibition was greater than 90%. 3-Aminobenzamide and 3-methoxybenzamide were both found to be competitive inhibitors, with K_i values (means ± S.E.M. of four experiments) of 1.8 ± 0.2μM and 1.5 ± 0.3μM respectively (results not shown). The greater degree of inhibition by the benzamides than by nicotinamide is not readily explained on the basis of simple structural or electron properties of the molecules.

3-Aminobenzamide was chosen to determine the effect of altering the carboxamide moiety on the ability of such compounds to inhibit the synthetase. It was surprising to find that both 3-aminobenzoic acid and 3-aminoacetophenone were inhibitory, although the inhibition was much less than with any of the benzamides (Tables 1 and 2). The corresponding nicotinamide analogues, nicotinic acid and 3-acetylpyridine, had no effect on enzyme activity (Table 2, and Preiss *et al.*, 1971). In contrast, alkylation of the amide group in both nicotinamide (Preiss *et al.*, 1971) and 3-aminobenzamide (to give 3-amino-*N*-methylbenzamide; Table 2) abolished inhibition. At present the inhibition by 3-aminobenzoic acid cannot be explained. It appears to be specific for 3-aminobenzoic acid; 3-nitro-, 3-hydroxy-, 2-amino- and 4-amino-benzoic acids all have no effect on enzyme activity. 3-Aminobenzoic acid has been shown to inhibit NAD$^+$ glycohydrolase (EC 3.2.2.5) by formation of an adduct with the NAD$^+$ substrate (Guardiola *et al.*, 1957). Such a mechanism cannot account for the inhibition of poly(ADP-ribose) synthetase, because neither 2-amino- nor 4-amino-benzoic acid (equally potent inhibitors of NAD$^+$ glycohydrolase) has any affect on synthetase activity. Furthermore, the lack of inhibition by the 2- and 4-isomers also precludes the possibility that inhibition by 3-aminobenzoic acid is by nucleophilic removal of ADP-ribose residues from the protein. Inhibition by 3-aminoacetophen-

Table 1. *Effect of various benzamides on poly(ADP-ribose) synthetase activity*

Enzyme activity was assayed as described in the Materials and Methods section. Inhibitors were added to a final concentration of 50μM (equimolar with substrate). Each value is the mean of at least four separate determinations.

Compound	NAD$^+$ incorporated into acid-insoluble material (nmol/min per mg of protein)	Inhibition (%)
None	2.40	—
Benzamide	0.09	96
3-Aminobenzamide	0.23	90
3-Bromobenzamide	0.12	95
3-Hydroxybenzamide	0.09	96
3-Methoxybenzamide	0.06	98
3-Nitrobenzamide	0.67	71
Nicotinamide	0.88	63

Table 2. *Effect of analogues of 3-aminobenzamide on poly(ADP ribose) synthetase activity*
Enzyme activity was assayed as described in the Materials and Methods section. Inhibitors were added to a final concentration of 50μM (equimolar with substrate). Each value is the mean of at least four separate determinations.

Compound	NAD^+ incorporated into acid-insoluble material (nmol/min per mg of protein)	Inhibition (%)
None	2.40	—
3-Aminobenzoic acid	2.16	10
Nicotinic acid	2.43	0
3-Hydroxybenzoic acid	2.38	0
3-Nitrobenzoic acid	2.45	0
2-Aminobenzoic acid	2.59	0
4-Aminobenzoic acid	2.47	0
3-Aminoacetophenone	1.66	31
3-Acetylpyridine	2.47	0
Acetophenone	1.53	36
3-Amino-*N*-methylbenzamide	2.38	0
3-Acetamidobenzamide	0.04	98
3-Succinylaminobenzamide	0.22	>91

one is less specific, as shown by the finding that acetophenone also inhibits (for a more detailed study on inhibition by acetophenones, see Purnell & Whish, 1980).

It was noteworthy that inhibition was retained when the amino group was acylated, as in 3-acetamidobenzamide and 3-succinylaminobenzamide. An accurate value could not be obtained for the latter compound, owing to its extremely low solubility. In addition, because detoxification of compounds containing aromatic amino groups often occurs by acetylation, 3-aminobenzamide detoxification will not lead to loss of inhibitor potency. Finally, coupling via the amino group should provide a very useful means by which an affinity medium for purification of the enzyme can be obtained.

In conclusion, the inhibitors described above will be useful for studies *in vitro* and, more importantly, since two, 3-aminobenzamide and 3-methoxybenzamide, are physiologically specific (M. R. Purnell & W. J. D. Whish, unpublished work), they will be extremely valuable as probes for use as inhibitors of poly(ADP-ribose) synthetase *in vivo*.

We thank the Science Research Council and Miles Laboratories, Stoke Poges, Slough, U.K., for their financial support.

References

Clark, J. B., Ferris, G. M. & Pinder, S. (1971) *Biochim. Biophys. Acta* **238**, 82–85
Finan, P. A. & Fothergill, G. A. (1961) *J. Chem. Soc.* 2824–2825
Guardiola, A. L., Paretsky, D. & McEwan, W. (1957) *J. Am. Chem. Soc.* **80**, 418–421
Hayaishi, O. & Ueda, K. (1977) *Annu. Rev. Biochem.* **46**, 95–116
Hilz, H. & Stone, P. R. (1976) *Rev. Physiol. Biochem. Pharmacol.* **76**, 1–58
Khan, M. G. & Shall, S. (1976) *Biochem. Soc. Trans.* **4**, 778–781
Leiber, U., Kittler, M. K. & Hilz, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1347–1350
Levi, V., Jacobson, E. L. & Jacobson, M. K. (1978) *FEBS Lett.* **88**, 144–146
Meuth, M., Aufreite, E. & Reichard, P. (1976) *Eur. J. Biochem.* **71**, 39–43
Preiss, J., Schlaeger, R. & Hilz, H. (1971) *FEBS Lett.* **19**, 244–246
Purnell, M. R. & Whish, W. J. D. (1980) *Biochem. Soc. Trans.* **8**, 175–176
Purnell, M. R., Stone, P. R. & Whish, W. J. D. (1980) *Biochem. Soc. Trans.* **8**, 215–227
Radziszewski, B. (1885) *Ber. Dtsch. Chem. Ges.* **18**, 355–356
Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552
Shall, S. (1975) *J. Biochem. (Tokyo)* **77**, 2p

of Rickwood *et al.* (1977)] gave excellent separation of ADP-ribosylated proteins from DNA and RNA. The material was precipitated by addition of trichloroacetic acid to a final concentration of 25% (w/v), left on ice for 4h, then washed and centrifuged three times at 38000*g* for 15min. The resulting material was digested with the following: (*a*) snake-venom phosphodiesterase. EC 3.1.4.1; (*b*) snake venom phosphodiesterase plus alkaline phosphatase, EC 3.1.3.1; (*c*) spleen phosphodiesterase. EC 3.1.4.18; (*d*) ribonuclease A, EC 3.1.4.22; (*e*) deoxyribonuclease I; EC 3.1.4.5; (*f*) Pronase (Hilz *et al.*, 1975); (*g*) base (Adamietz *et al.*, 1978); (*h*) hydroxylamine (Adamietz *et al.*, 1978). Increase in radioactivity in the supernatant of a 10min, 10000*g* centrifugation in 66% (v/v) ethanol/50mM-sodium acetate, pH5.0, gave an indication of the amount of acid-insoluble material digested. Results of this preliminary characterization showed that 92% was solubilized with snake-venom phosphodiesterase, 17% with spleen phosphodiesterase, 14% with ribonuclease, 0% with deoxyribonuclease, 68% with Pronase, 87% with base, and 21% with hydroxylamine. This digested material was then subjected to a number of t.l.c. systems. Unambiguous determination of poly(ADP-ribose) was made by digestion with snake-venom phosphodiesterase, which gives rise to phosphoribosyl-AMP, a unique marker for poly(ADP-ribose), which was identified by t.l.c. on polyethyleneimine-cellulose (K. Randerath & E. Randerath, 1965). Phosphoribosyl-AMP was further characterized by digestion with alkaline phosphatase to give ribosyladenosine and identified by t.l.c. (Miwa *et al.*, 1979). The above two studies gave an average chain length of polymer attached to protein of 1.2 ADP-ribose units. The linkage between poly(ADP-ribose) and protein has been reported to be sensitive to both base and hydroxylamine (Nishizuka *et al.*, 1969). T.l.c. analysis of the transient products of these chemical digests showed that ADP-ribose was indeed produced.

Digestion of gradient material overnight with 0.2M-NaOH will produce 5'-AMP from monomer, 3'-AMP from RNA, but will not digest polymer. The results of two different t.l.c. systems (Schwartz & Drach, 1975; E. Randerath & K. Randerath, 1965) indicated that, in fact, 15% of material was present as 3'-AMP, 55% as 5'-AMP and 20% remained on the origin as polymer. Further digestion of the base-treated material with 3'-nucleotidase (EC 3.1.3.6) and 5'-nucleotidase (EC 3.1.3.5), followed by determination of percentage decrease in AMP and increase in adenosine on t.l.c. gave similar values, which corresponded well with those of the enzymic digests with respect to both amount of RNA and the polymer/monomer ratio.

The possibility of DNA contamination was eliminated by an exhaustive digest with snake-venom phosphodiesterase, deoxyribonuclease I and alkaline phosphatase, followed by a borate t.l.c. system capable of distinguishing adenosine from deoxyadenosine (K. Randerath & E. Randerath, 1965). This analysis showed the complete absence of deoxyadenosine and therefore of DNA. It was then possible to carry out further analysis of the properties of the system *in vivo*. It has been well established that a number of inhibitors exist capable of inhibiting poly(ADP-ribose) polymerase *in vitro*, including thymidine (Preiss *et al.*, 1971). Thus cells were taken 6h before labelling and incubated with 5mM-thymidine, 2mM-3-aminobenzamide or 2mM-3-methoxybenzamide, the latter two being inhibitors that have been developed in this laboratory and found to produce an even higher percentage inhibition than others previously described (M. R. Purnell & W. J. D. Whish, unpublished work). However, with these inhibitors only 10% inhibition of the ADP-ribosylation of protein was found. Chain-length studies revealed that although the radioactivities in AMP were the same, there was no detectable phosphoribosyl-AMP. This provides the interesting possibility that the inhibitors work at the polymerization step but not at the initiation step. It is therefore possible that more than one enzyme exists, one for initiation and one for polymerization.

It thus appears that the analysis of ADP-ribosylation *in vivo* can provide much insight into understanding the role of this complex system.

We are grateful to the Medical Research Council for support.

Adamietz, P., Bredehorst, R., Hilz, H. (1978) *Eur. J. Biochem.* **91**, 317–326
Hayaishi, O. & Ueda, K. (1977) *Annu. Rev. Biochem.* **46**, 95–116
Hilz, H. & Stone, P. R. (1976) *Rev. Physiol. Biochem. Pharmacol.* **76**, 1–58
Hilz, H., Weigers, U., Adamietz, P. (1975) *Eur. J. Biochem.* **56**. 103–108
Miwa, M., Saikawa, N., Yamaizumi, Z., Nishimura, S. & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 595/599
Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M. & Hayaishi, O. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 781–786 .
Preiss, J., Schaeger, R., Hilz, H. (1971) *FEBS Lett.* **19**, 244–246
Randerath, E. & Randerath, K. (1965) *Anal. Biochem.* **12**, 83–93
Randerath, K. & Randerath, E. (1965) *Anal. Biochem.***13**, 575–579
Rickwood, D., MacGillivray, A. J. & Whish, W. J. D. (1977) *Eur. J. Biochem.* **79**, 589–598
Schwartz, P. M. & Drach, J. C. (1975) *J. Chromatogr.* **106**, 200–203

The effect of phenones on poly(adenosine diphosphate ribose) synthetase from porcine thymus

MICHAEL R. PURNELL and
WILLIAM J. D. WHISH
Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

Poly(ADP-ribose) synthetase is a nuclear enzyme responsible for the covalent modification of proteins by ADP-ribose or an oligomer thereof. NAD is cleaved at the nicotinamide–ribose bond and the ADP-ribose is attached to either a protein or a protein-bound $(ADP\text{-}ribose)_n$ chain, where $n = 1–30$.

The biological function of this enzyme remains unknown. It has been postulated that the enzyme may be involved in DNA synthesis, transcription or repair (Hilz & Stone, 1976). During a recent study involving the screening of benzamide molecules as potentially specific inhibitors of the enzyme, it was found that acetophenone and 3-aminoacetophenone were inhibitory (M. R. Purnell & W. J. D. Whish, unpublished work). Nicotinamide is known to inhibit the enzyme, and the binding site is highly specific (Priess *et al.*, 1971). The effect of several phenones on the enzyme was examined to see whether the same specificity was required for inhibition by analogues of acetophenone.

Poly(ADP-ribose) synthetase was extracted from porcine thymus nuclei, isolated as described by Khan & Shall (1976), with 0.5M-NaCl/100mM-triethanolamine/HCl (pH8.2)/10mM-$MgCl_2$/2.5mM-dithiothreitol. The enzyme activity was determined by the incorporation of $[^3H]NAD^+$ into acid-insoluble material. The reaction mixture contained 100mM-triethanolamine/HCl, pH8.2, 10mM-$MgCl_2$, 2.5mM-dithiothreitol, 50μM-[^{3}H]NAD (sp. radioactivity 2mCi/mmol) in 180μl. The reaction was started by the addition of 20μl of 0.5M-NaCl extract.

Table 1. *Effect of acetophenone and its analogues on poly(ADP-ribose) synthetase*

Enzyme activity was determined as described in the text. Each value is the mean of four separate determinations.

Addition (50 μM)	10^{-4} x Radioactivity incorporated (c.p.m./5 min per 50 μg of protein)	Inhibition (%)
None	7.5	—
Acetophenone	4.7	36
3-Aminoacetophenone	5.3	30
3-Bromoacetophenone	4.4	42
3-Hydroxyacetophenone	3.9	47
3-Methoxyacetophenone	3.3	56
3-Nitroacetophenone	7.2	3
Propiophenone	7.0	6
$\alpha\alpha\alpha$-Trifluoroacetophenone	6.9	7
4-Nitroacetophenone	7.1	5
Menadione	5.7	24

After 5 min at 26°C the radioactive acid-insoluble material was determined by addition of 20 μl of the reaction mixture to a trichloroacetic acid-impregnated filter disc. The disc was left in 20% (w/v) trichloroacetic acid for 30 min at 0°C. After being washed three times in 5% (w/v) trichloroacetic acid for 5 min, the disc was washed briefly in ethanol and then diethyl ether. After the disc was dried at room temperature, the radioactivity was determined by liquid scintillation counting in 0.5% (w/v) PPO (2,5-diphenyloxazole) in toluene.

Table 1 shows the effect of various compounds at 50 μM (equimolar with substrate) on enzyme activity. Acetophenone inhibits the enzyme 36%. Substitution of the aromatic nucleus at the 3-position increases the potency of inhibition to a slight extent, with the exception of 3-nitroacetophenone, which exerts a negligible effect on the enzyme. As in the case of benzamides, the 3-methoxy derivative is the most inhibitory (M. R. Purnell & W. J. D. Whish, unpublished work). Alteration of the acetyl group as in the case of propiophenone and trifluoroacetophenone results in complete loss of inhibition.

In view of the proposed role of poly(ADP-ribose) in the repair of DNA and especially the enhancement of the cytotoxic effect of DNA-damaging agents by inhibitors of poly(ADP-ribose) synthetase (Shall *et al.*, 1977), the effects of two radiosensitizers on enzyme activity were examined. 4-Nitroacetophenone had no effect on the enzyme. Menadione or Synkay (2-methylnaphthoquinone), however, inhibited the enzyme 24% at 50 μM. The radiosensitization exhibited by both compounds has been ascribed to their electron-affinic properties (Adams & Cooke, 1969). In the case of menadione, the radiosensitization may be due, in part, to inhibition of poly(ADP-ribose) synthetase and a concommitant deficiency in DNA repair.

From this observation, and the data of Shall *et al.* (1977), it would appear that poly(ADP-ribose) synthetase may be a useful target molecule for enhancing the cytotoxic effects of ionizing radiation and other DNA-damaging agents.

We thank the Science Research Council and Miles Laboratories (Stoke Poges, Slough, Berks., U.K.) for their support throughout this work.

Adams, G. E. & Cooke, M. S. (1969) *Int. J. Rad. Biol. Relat. Stud. Phys. Chem. Med.* **15**, 457–471
Hilz, H. & Stone, P. R. (1976) *Rev. Physiol. Biochem. Pharmacol.* **76**, 1–58
Khan, M. G. & Shall, S. (1976) *Biochem. Soc. Trans.* **4**, 778–781
Priess, J., Schlaeger, R. & Hilz, H. (1971) *FEBS Lett.* **19**, 244–246
Shall, S., Goodwin, P., Halldorsson, H., Khan, G., Skidmore, C. & Tsopanakis, C. (1977) *Biochem. Soc. Symp.* **42**, 103–116

Computer-assisted sequencing of peptide mass spectra

CHRISTINE THIRKETTLE and
HOWARD R. MORRIS

Department of Biochemistry, Imperial College, South Kensington, London SW7, U.K.

Procedures have been developed for the mass-spectrometric sequencing of peptides (Morris *et al.*, 1974, 1976; Dell & Morris, 1977) and are now in regular use in this Laboratory. These methods have two basic advantages over classical procedures: firstly a considerable saving in time because of the capability of studying mixtures of peptides, and secondly a capacity to assign blocked sequences (natural *N*-acetyl or cyclized Glx) or new/unusual amino acids. The time saving associated with not having to purify each peptide amounts to approximately one-half of the time normally taken to sequence a protein. However, a new rate-limiting step arises in the mass-spectrometric method, and if this could be removed or reduced then it would revolutionize the sequencing process, allowing the generation of a 25000-dalton sequence in some 2–3 man months. We here report a step towards this goal in the development of a semi-automated interactive sequencing procedure.

In order to cut the time taken to count and interpret the large number of spectra of peptide mixtures generated when a protein or large peptide is being sequenced, computer programs have been written to assist in the interpretation. The computer method follows a strategy similar to that employed by the spectroscopist. The path taken by the programs with any particular set of spectra is directed by the operator, who is given a number of possibilities from which to choose at each point in the sequencing process. The programs sequence low-resolution mass data starting from the *N*-termini and N–C cleavage ions, and proceeding towards the *C*-terminus of each peptide. In choosing the next sequence ions, due consideration is given to losses of side chains from serine, methionine and threonine, loss of CO from residues after N–C cleavage ions, pyrrolid-2-one-5-carboxylic acid from glutamate or glutamine and other indicators of specific amino acids, e.g. *m/e* 171 for lysine. A number of scans can be analysed together, in order to compare spectra run at different source temperatures, to facilitate the examination of peptide mixtures. Reaction-condition parameters can be selected for the interpretation of the spectra of sample derivatives produced by any combination of acetylation, deuteroacetylation, permethylation and perdeuteromethylation.

There are two separate programs. In the pre-processing program scans previously stored on disc by the data-aquisition software are selected and their data converted into a format more readily usable for sequencing. In the sequencing program the user chooses up to four pre-processed scans before going on to select from the list of options available to direct the actual sequencing. These options can be run or repeated in any order and enable the spectroscopist to build a table of peptide sequences found: sequence ions can be added to or deleted from this table.

The options available are:

(1) Display graphically the region of the spectrum containing the *N*-terminal and N–C cleavage ions for the current main scan, with a subsidiary scan superimposed in bar form on this scan. Present the user with possible *N*-termini from the current main scan for incorporation into the table.

Research Overview

ADP-ribosylation of nuclear proteins

MICHAEL R. PURNELL, PETER R. STONE and WILLIAM J. D. WHISH

Biochemistry Group, School of Biological Sciences, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

ADP-ribosylation can be defined as the postsynthetic modification of protein by the covalent attachment of the ADP-ribose moiety of NAD^+. ADP-ribosylation of elongation-factor Tu is responsible for the inhibition of protein synthesis by both diptheria and *Pseudomonas aeroginosa* toxins (Hilz & Stone, 1976). The activation of membrane adenylate cyclase by cholera toxin is thought to occur by ADP-ribosylation of the adenylate cyclase-associated GTP binding protein (Gill & Meren, 1978). It has also been proposed as a mechanism by which *Escherichia coli* RNA polymerase activity is modified during bacteriophage T4 infection (Goff, 1974; Rohrer *et al.*, 1975; Skorko *et al.*, 1977). Coliphage N4 has also been reported to contain an intrinsic ADP-ribosyltransferase activity (Pesce *et al.*, 1976). ADP-ribosyltransferase activity is also present in the mitochondria and nuclei of all eukaryotic organisms examined to date.

The field of ADP-ribosylation has been comprehensively reviewed by Sugimura (1973), Hilz & Stone (1976) and Hayaishi & Ueda (1977). The present review is concerned exclusively with ADP-ribosylation carried out in eukaryotic nuclei and will concentrate on the large amount of data that has emerged subsequent to the publication of other reviews.

The enzyme responsible for ADP-ribosylation in nuclei is termed poly(ADP-ribose) synthetase or polymerase; the name is derived from the fact that, unlike other ADP-ribosyltransferases, the enzyme is capable of synthesizing a protein-bound homopolymer of ADP-ribose, poly(ADP-ribose) (Chambon *et al.*, 1966; Reeder *et al.*, 1967; Fujimura *et al.*, 1967). NAD is cleaved at the nicotinamide–ribose bond (bond energy ~ 34 kJ/mol) and the ADP-ribose moiety transferred to either a nuclear protein or a protein-bound ADP-ribose molecule. The polymer thus formed is degraded by another nuclear enzyme, poly(ADP-ribose) glycohydrolase, producing the free monomeric form of ADP-ribose (Miwa & Sugimura, 1971) (Fig. 1).

(1) *ADP-ribosylation of nuclear proteins*

(a) Structure of mono- and poly-(ADP-ribose)–protein conjugates. ADP-ribose is attached to proteins through the free ribose molecule by two distinct types of bonds, and the observation has been made that, after incubation of isolated rat liver nuclei with NAD radioactively labelled in the adenine moiety, all radioactivity is removed by dilute alkali, but only 60% is removed by neutral hydroxylamine (Adamietz & Hilz, 1976). Endogenous mono(ADP-ribose) residues linked to proteins isolated from Ehrlich ascites-tumour cells and rat liver have also been reported to possess similar labilities (Bredehorst *et al.*, 1978*c*), although the relative proportions of each type of linkage are different [see Section (1)(*c*) below] in the two tissues. More recently, it has been suggested that a third, alkali-resistant, bond may exist *in vivo* (Adamietz *et al.*, 1978*a*). It should be mentioned here that poly(ADP-ribose) is stable in alkali (Fujimura *et al.*, 1967; Nishizuka *et al.*, 1967).

Proposals as to the nature of the linkage to protein vary from a carboxylic acid ester (Nishizuka *et al.*, 1968, 1969), a Schiff base with lysine (Kun *et al.*, 1976), or a phosphodiesterase linkage with phosphoserine (Smith & Stocken, 1973, 1975). The carboxylic acid ester was proposed to account for the lability towards hydroxylamine. It has since been reported that glutamic acid residues at position 2 are ADP-ribosylated in both histones H1 and H2B (Burzio *et al.*, 1979; Riquelme *et al.*, 1979). Hayaishi *et al.* (1979) have also reported ADP-ribosylation at these positions and, in addition, at positions 4 and 116 in histone H1. Both groups used isolated nuclei from rat liver.

The chemical structure of poly(ADP-ribose) was first elucidated by Doly (Chambon *et al.*, 1966). The anomeric carbon of one ADP-ribose molecule was found to be attached to the adenosine moiety of the next via a 1″–2′ glycosidic linkage. Subsequent n.m.r. analyses of either pure poly(ADP-ribose) (Miwa *et al.*, 1977*b*) or its degradation product, 2′-(5″-phosphoribosyl)-5′-AMP (Miwa *et al.*, 1977*b*; Ferro & Oppenheimer, 1978; Inagaki *et al.*, 1978), have identified the linkage as α-(1″–2′), as shown in Fig. 1.

Recent studies on the chain length of poly(ADP-ribose) formed in isolated nuclei show the product may have as many as 65 ADP-ribose residues. The earliest method of determining the chain length was by incubation of isolated nuclei with radioactively labelled NAD, digestion of the product with snake venom phosphodiesterase, resolution of 5′-AMP and phosphoribosyl-AMP by paper chromatography and analysis of the radioactivity associated with each compound. The ratio of the total radioactivity (AMP and phosphoribosyl-AMP) to the radioactivity in AMP gives an average chain length (Nishizuka *et al.*, 1969). The first method of resolving poly(ADP-ribose) of differing chain lengths was that of Sugimura *et al.* (1971). Isolated nuclei labelled with [^{14}C]NAD were digested with Pronase and the poly(ADP-ribose) and nucleic acids were precipitated with ethanol. The pellet was resuspended and subjected to hydroxyapatite column chromatography; elution was effected with increasing concentrations of phosphate buffer. In addition to separation of nucleic acids from poly(ADP-ribose), they observed a linear relationship between chain length and phosphate concentration. The same group later analysed each of the observed peaks by gel electrophoresis and found each peak comprised two subfractions differing in both chain length and terminal structure (Tanaka *et al.*, 1977). They suggested that the latter may be due to partial degradation by hydrolytic enzymes.

Adamietz *et al.* (1978*b*) used polyacrylamide-gel electrophoresis to separate poly(ADP-ribose) molecules of differing chain lengths after alkaline digestion of labelled isolated nuclei. Discrete bands corresponding to poly(ADP-ribose) of up to 33 residues in length were detected by fluorography. They were able to show quantitative differences in the chain-length pattern from nuclei isolated from different tissues by determining the radioactivity in each band.

A similar protocol was used by Tanaka *et al.* (1978) to resolve various fractions of poly(ADP-ribose) after hydroxyapatite column chromatography. They were able to show the presence of at least 65 discrete bands. After analysing the chain length of each band component by the method of Nishizuka *et al.* (1969), they found an increasing chain length up to $(ADP\text{-}ribose)_{30}$. Bands corresponding to chain lengths above this, however, showed no increased chain length by the phosphodiesterase method. They suggested that this could be due to the pres-

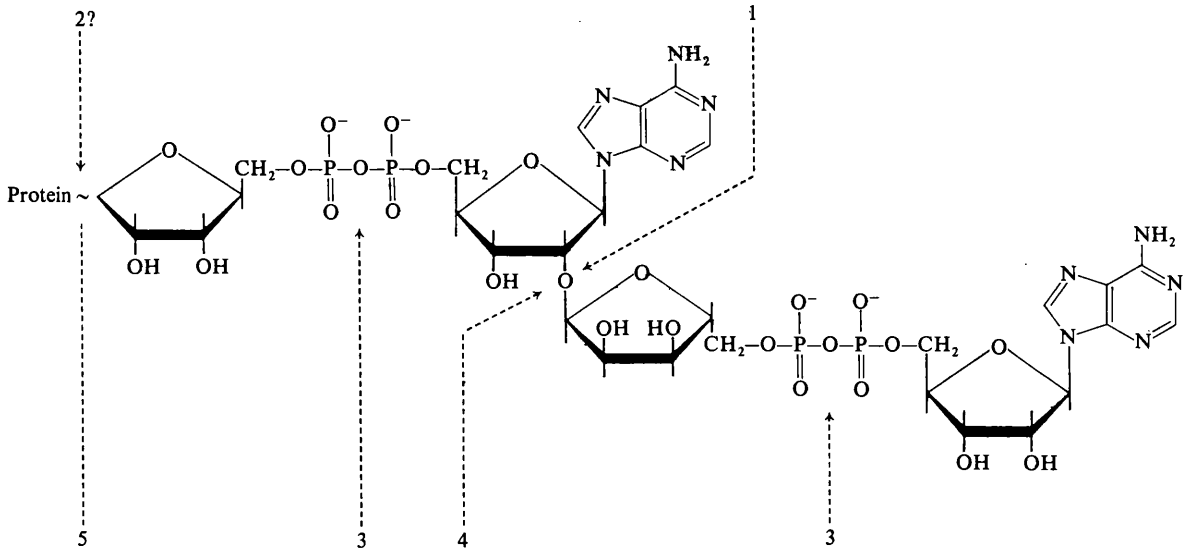


Fig. 1. *Enzymology of ADP-ribosylation*

1, Poly(ADP-ribose) synthetase; 2, initiating enzyme; 3, phosphodiesterase; 4, poly(ADP-ribose) glycohydrolase; 5, ADP-ribose–protein hydrolase.

ence of a branched polymer having more than one AMP terminus. Recently, Miwa *et al.* (1979) have isolated and identified a branched structure whereby additional ADP-ribose molecules are attached to poly(ADP-ribose) via a 1‴–2″ or a 1‴–3″ glycosidic linkage with the ribose to which the nicotinamide was originally attached.

Farzaneh & Pearson (1978) used the hydroxyapatite-column-chromatography method to determine the chain length of poly(ADP-ribose) during development of the toad *Xenopus laevis*. In all cases they found this method gave larger estimates than the phosphodiesterase method of Nishizuka *et al.* (1969). They proposed that [^{3}H]NAD was incorporated *in vitro* on to pre-existing chains synthesized *in vivo*.

(*b*) *Identification of proteins modified in vitro.* Two general approaches have been adopted to identify protein acceptors *in vitro*. The first method consists of isolating specific proteins from nuclei labelled with radioactive NAD. This method is restricted to the relatively well defined proteins such as histones. In this manner several laboratories have shown histones H1 and H2B to be the major acceptors amongst the histones (see Hilz & Stone, 1976; Hayaishi & Ueda, 1977). Other acceptors that have been identified are high-mobility-group (HMG) proteins, trout specific H6 protein and protamines (Wong *et al.*, 1977). Tanuma *et al.* (1977) studied the ADP-ribosylation of histones in nuclei isolated from HeLa cells. On extraction of histone H1 with $HClO_4$ and acetic acid/urea/polyacrylamide-gel electrophoresis, the bulk of the radioactivity was seen to migrate more slowly than histone H1. Stone *et al.* (1977) have reported a similar result and have characterized this slower-moving material as a dimer complex consisting of two histone H1 molecules joined by a single chain of poly(ADP-ribose) of length 15 ADP-ribose units. The significance of such a modification will be discussed in Section (3)(*h*).

A broader study of poly(ADP-ribosyl)ation has been presented by Rickwood *et al.* (1977). [^{32}P]ADP-ribosylated proteins were separated from nucleic acids by CsCl-equilibrium-density-gradient centrifugation, fractionated on hydroxyapatite and subjected to two-dimensional gel electrophoresis. Subsequent radioautography showed that more than 30 protein species were modified. Proteins bearing various amounts of ADP-ribose were resolved by this method.

A new approach for the separation of ADP-ribosylated proteins from unmodified proteins was recently developed by Okayama *et al.* (1978*a*). The method uses complex-formation between the *cis*-2′,3′-diol of the 'nicotinamide' ribose of the ADP-ribose and immobilized aminophenylboronic acid. Among the proteins shown to be modified are histones H1, H2A, H2B and high-mobility-group (HMG) proteins (Hayaishi *et al.*, 1979), and A24 protein (Okayama & Hayaishi, 1978).

The second method used to determine if a protein is modified has been to incubate the protein in a reconstituted system with purified poly(ADP-ribose) synthetase, DNA and labelled NAD. In this manner, a (Ca^{2+} + Mg^{2+})-dependent endonuclease (Yoshihara *et al.*, 1975), and poly(ADP-ribose) synthetase itself (Yoshihara *et al.*, 1977) have been shown to act as acceptors. However, when Okayama *et al.* (1977) used histones in a similar preparation, they could not detect any radioactivity associated with any stainable protein bands after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The bulk of the radioactivity ran with an R_F of 0.58. Ellison (1978) found the same result using a similar method; it was subsequently found that the peak was present when the enzyme preparation was omitted or when labelled NAD alone was analysed.

(*c*) *Identification and quantification of mono- and poly-(ADP-ribosyl)ated proteins modified in vivo.* The identification of ADP-ribosylated proteins and the extent of their modification during various functional states of a cell is crucial to understanding the physiological significance of this modification. Clarification of these problems presents workers with several major difficulties. No suitable precursor exists for labelling ADP-ribosylated proteins *in vivo*. Use of adenine, ribose or phosphate necessitates removal of contaminants such as nucleic acids, phosphoproteins or glycoproteins. Removal must be complete, since amounts of ADP-ribose–protein conjugates appear to be much less than 1% of the amount of DNA or RNA. In addition, the lability of the linkage to proteins at neutral or alkaline pH means conditions must be carefully controlled. Also, it has been suggested that turnover is extremely rapid, possibly less than 5 min (Benjamin & Gill, 1979). Solution of these problems has been achieved by exploiting a number of properties that in combination are unique to ADP-ribose and poly(ADP-ribose). That is, they are covalently attached to protein, possess (a) pyrophosphate bond(s) and in the case of poly(ADP-ribose) contain a 1″–2′ glycosidic linkage.

ADP-ribosylation of histones has been studied by extraction with inorganic acid of cells labelled *in vivo* and resolving the proteins present. Ueda *et al.* (1975) showed that histones H1, H2 and H3 are modified, and Ord & Stocken (1975) showed that histone H1, a protein P1 and a small peptide were all modified; both groups used rat liver. More recently, Adamietz *et al.* (1978*a*) used a different extraction procedure to show that histone H1 was ADP-ribosylated in HeLa cells by both mono- and poly-(ADP-ribose). In contrast with the studies *in vitro* by Stone *et al.* (1977), they could not detect histone H1 dimer.

Various groups have used isotopic methods to show qualitative changes in the amount of mono- and poly-(ADP-ribose) under various cell conditions. Colyer *et al.* (1973) showed differences in concentrations of poly(ADP-ribose) during the cell cycle of synchronized L-cells. After pulse-labelling with [^{3}H]adenosine, nuclei were isolated and treated with alkali. DNA and protein were removed by high-speed centrifugation and [^{3}H]-labelled poly(ADP-ribose) in the supernatant was recovered by acid precipitation. Poly(ADP-ribose) was then hydrolysed in $HClO_4$ at 90°C and the radioactivity determined. Ghani & Hollenberg (1978*b*) used a similar approach to show differences in poly(ADP-ribose) in chick-embryo heart cells after growth in 5 and 20% O_2. Cells were labelled with [^{14}C]ribose and treated with acid to precipitate macromolecules. After washing, the pellet was treated with alkali, digested with deoxyribonuclease and ribonuclease, followed by centrifugation and then the supernatant deproteinized with chloroform/3-methylbutan-1-ol mixture. The [^{14}C]poly(ADP-ribose) was recovered from the aqueous layer by addition of ethanol. Dietrich *et al.* (1973) used nuclei isolated from rat livers labelled *in vivo* with [^{32}P]P_i to show relative amounts of hydroxylamine-sensitive and -resistant ADP-ribosylated proteins. After treatment of nuclei with trichloroacetic acid and washing, the pellet was suspended in 0.4 M-hydroxylamine, pH 7.0, for 1 h at 0°C. The mixture was centrifuged and the supernatant then treated with acid and centrifuged again. The supernatant from this was analysed by ion-exchange and thin-layer chromatography, which showed that 11% of the recovered radioactivity corresponded to ADP-ribose and 3% to oligo(ADP-ribose). The pellet was digested with Pronase, deoxyribonuclease and ribonuclease and the products analysed by hydroxylapatite chromatography (section 1*a*). The material eluting at the same position as authentic poly(ADP-ribose) was hydrolysed with snake-venom phosphodiesterase. Phosphoribosyl-AMP could not be detected by t.l.c. analysis, which suggests that no poly(ADP-ribose) was present in this fraction, although it is possible that degradation occurred during the isolation procedure.

All the methods described above were designed to show qualitative changes of ADP-ribosylated proteins. Described below are methods that attempt to quantify amounts *in vivo*.

Stone *et al.* (1976) used an isotope-dilution method to quantify poly(ADP-ribose) in various rat tissues. [^{3}H]adenosine-labelled poly(ADP-ribose) of known specific radioactivity was synthesized *in vitro*, and then added to a homogenate of the rat tissue. After treatment with alkali and hydroxylamine the poly(ADP-ribose) was partially purified by ion-exchange chromatography. The digestion product from snake-venom phosphodiesterase, phosphoribosyl-AMP, was partially purified by paper chromatography and after digestion with alkaline phosphatase, ribosyladenosine was purified to constant specific radioactivity. From this, and the known starting specific activity, the amount of unlabelled (*in vivo*) poly(ADP-ribose) was calculated to be 5.59 nmol/mg of DNA for adult and 6.32 nmol/mg of DNA in neonatal-rat liver. Mono(ADP-ribose), determined by a similar method (Stone & Hilz, 1975) gave values of 5.28 and 2.19 nmol of ADP-ribose/mg of DNA for adult and neonatal-rat liver respectively.

An optical test was developed by Goebel *et al.* (1977) to quantify hydroxylamine-sensitive and -resistant mono(ADP-ribosyl)ated proteins in rat liver and Ehrlich ascites-tumour cells. The method relies on the release of 5′-AMP, as a unique product, from ADP-ribose or mono(ADP-ribosyl)ated ADP–proteins upon treatment with alkali. 5′-AMP was quantified by using a linked enzyme assay. Hydroxylamine-sensitive mono(ADP-ribose) is present in Ehrlich Ascites Tumour cells at approx. 1 nmol/mg of DNA and does not appear to vary throughout the growth cycle. The authors reported that amounts of poly(ADP-ribose) could be determined by the method after digestion with poly(ADP-ribose) glycohydrolase. The chain length of the polymer could also be determined by measuring the ratio of nmol of ADP-ribose released by poly(ADP-ribose) glycohydrolase to nmol of 5′-AMP released by phosphodiesterase. Although no values were reported for material *in vivo*, the method gave comparative values for poly(ADP-ribose) synthesized *in vitro* with values obtained by the method of Nishizuka *et al.* (1969).

Two groups have used fluorimetric assays to determine amounts of poly(ADP-ribose) *in vivo*. Niedergang *et al.* (1978) treated rat liver isolated nuclei with alkali and purified the released poly(ADP-ribose) by a four step procedure. After enzymic hydrolysis, either ADP-ribose or phosphoribosyl-AMP were quantified fluorimetrically by the glyoxal method of Yuki *et al.* (1972). Sims *et al.* (1979) reported a different method based on the dihydroxyborate column of Okayama *et al.* (1978*a*). Poly(ADP-ribose) from crude cellular extracts of 3T3 cells was purified by this method, and after digestion with snake-venom phosphodiesterase and alkaline phosphatase; ribosyladenosine was treated with chloroacetaldehyde. After purification by high-pressure liquid chromatography the derivative was quantified. Niedergang *et al.* (1978) measured values of 3.35 nmol/mg of DNA for adult rat liver. Sims *et al.* (1979) reported no values for the amount of poly(ADP-ribose).

Antibodies against poly(ADP-ribose) have been used as a basis for determining amounts *in vivo* by radioimmunology. The first report of antibodies raised against poly(ADP-ribose) was by Kanai *et al.* (1974). The final preparation was highly specific and could detect poly(ADP-ribose) at 3 μg/ml. The same authors later measured the amounts of poly(ADP-ribose) in calf thymus to be 0.02 μg/mg of DNA (Sakura *et al.*, 1977). They also showed that anti-[poly(ADP-ribose)] antibodies were present in the sera of patients with the autoimmune disease systemic lupus erythmatosus (Kanai *et al.*, 1977). Okolie & Shall (1979) suggest that such antibodies may provide a useful diagnostic test for the disease. Antibodies to poly(ADP-ribose) were raised in response to injected poly(A)·poly(U) (Kanai *et al.*, 1978) and a phosphoribosyl-AMP–bovine serum albumin conjugate (Sakura *et al.*, 1978). No quantitative data has been reported with these antibodies. Kidwell & Mage (1976) used antibodies against poly(ADP-ribose) to evaluate amounts during the cell cycle of HeLa cells. The maximum amount was 83 ng/mg of DNA. Ferro *et al.* (1978) used antibodies against poly(ADP-ribose)$_{40}$ to determine amounts of poly(ADP-ribose) in various rat and pigeon tissues. Nuclei were isolated by an organic-solvent technique, thereby minimizing the effect of degradative enzymes. Amounts ranged from 58 ng/mg of DNA in rat liver to over 1 μg/mg of DNA in pigeon heart. It was noteworthy that treatment with nicotinamide, an inhibitor of poly(ADP-ribose) synthetase (section 1*d*), increased the amounts of poly(ADP-ribose) in rat liver and rat heart. This may be due to increased amounts of NAD in response to nicotinamide.

Bredehorst *et al.* (1978*a*,*b*) developed a radioimmunoassay based on antibodies to 5′-AMP to quantify protein-bound mono- and poly-(ADP-ribose) residues. The released mono(ADP-ribose) and ADP-ribose derived from polymer, after treatment with poly(ADP-ribose) glycohydrolase, was converted into 5′-AMP by treatment with NaOH and then assayed. Values obtained for the sensitive mono(ADP-ribose) residues in stationary-phase Ehrlich ascites-tumour cells and rat liver were 0.48 nmol/mg of DNA and 5.3 nmol/mg of DNA res-

pectively. Total NaOH released mono(ADP-ribose) residues were 1.66 nmol/mg of DNA for Ehrlich ascites-tumour cells and 1.26 nmol/mg of DNA for rat liver (Bredehorst *et al.*, 1979*c*).

All of the methods described above depend, at some stage, on the hydrolysis of the protein–ADP-ribose bond. Described below are two methods that rely on keeping this bond intact and that could thus be used for subsequent identification and quantification of modified proteins. Young & Sweeney (1978, 1979) were able to isolate ADP-ribosylated proteins from unfertilized mouse ova labelled with [^{3}H]adenosine. Since the ova were not actively synthesizing DNA and RNA during the labelling period, all the acid-insoluble radioactive material was present as either poly(A) tracts or ADP-ribosylated proteins. These could be isolated by removal of other polynucleotides by extraction with chloroform/phenol and digestion of the precipitated material with ribonucleases followed by precipitation of the protein. Acid-soluble nucleotides and nucleosides could be removed by washing, although the authors did report that some ATP was still retained.

Whish's group (Purnell *et al.*, 1980; Surowy & Whish 1980) used a modification of the density-gradient-centrifugation method of Rickwood *et al.* (1977) to remove nucleic acids from [^{3}H]adenosine-labelled whole-cell homogenates. The relative proportions of mono- and poly-(ADP-ribose) conjugates could be determined by t.l.c. on polyethyleneimine–cellulose after base hydrolysis. By omission of the alkali step, the proteins can be resolved by using methods used by Rickwood *et al.* (1977) and the [^{3}H]ADP-ribosylated proteins detected by fluorography.

(*d*) *Synthesis of poly(ADP-ribose).* Early attempts to purify poly(ADP-ribose) synthetase were summarized by Hilz & Stone (1976), and Table 1 lists the purification procedures that have been developed since that time. Khan & Shall (1976) were the first to attempt to use affinity chromatography. A purification of 85-fold was achieved by using a column containing nicotinamide and 34-fold with one containing Blue Dextran. In both cases the yield of enzyme activity was greater than 100%. This may be due to removal of degradative enzymes or, as the authors suggest, an endogenous inhibitor. Several published methods have since used similar steps. The affinity of the enzyme for DNA was first exploited by Kristensen & Holtlund (1976). A 131-fold purification was achieved by phosphate extraction and DNA–cellulose chromatography. An additional isoelectric-focusing step was later included (Kristensen & Holtlund, 1978) to achieve a final purification of 700-fold. Tsopanakis *et al.* (1976) and Ellison (1978) observed that the final enzyme preparation was extremely labile. To minimize inactivation during purification, Tsopanakis *et al.* (1977) later used an organic solvent [50% (*v*/*v*) ethylene glycol] at 4°C and at −10°C (Tsopanakis *et al.*, 1978*a*) to achieve higher purification and yield of enzyme activity.

The molecular weight of the enzyme has been shown to vary from 62000 in pig thymus (Tsopanakis *et al.*, 1978*a*) to 130000 in calf thymus (Yoshihara *et al.*, 1978). The most exhaustive characterization of poly(ADP-ribose) synthetase is that of Ito *et al.* (1979). The apparently homogeneous enzyme preparation had a mol.wt. of 110000. Sedimentation data suggests the enzyme is a globular protein with slight asymmetry. The enzyme has a pI of 9.8 and amino acid analysis showed more lysine than arginine residues. The *N*-terminus appears to be blocked; this is in agreement with the data of Tsopanakis *et al.* (1978*a*) for the pig thymus enzyme.

In addition to the use of purified enzyme preparations, studies on the synthesis of poly(ADP-ribose) in isolated nuclei or crude nuclear extracts have been used to characterize poly(ADP-ribose) synthetase. The enzyme requires Mg^{2+} and a thiol-containing reagent for maximal activity. The pH optimum is approx. 8 and the temperature optimum is usually some 10–15°C below the optional growing temperature of the cells or tissue from which the enzyme is isolated. The K_m for NAD^+ varies from 40 μM in quail oviduct to 1.5 mM in mouse L-cells (Hilz & Stone, 1976). The specificity for β-NAD is high; neither $NADH_2$ nor $NADP^+$ is incorporated. Various NAD analogues with altered adenine moieties are incorporated into monomer or polymer but at much slower rates than NAD^+ (Suhadolnik *et al.*, 1977). The enzyme is tightly bound to DNA, and purified preparations also have an absolute requirement for DNA (e.g. Ito *et al.*, 1979). Fragmented DNA stimulates synthesis more than native DNA. A recent study (Benjamin & Gill, 1978) shows that plasmid-PMB9 supercoiled DNA supported activity of a partially purified enzyme preparation. Activity was further stimulated when the DNA was 'nicked' once with *Eco*RI restriction endonuclease or greatly stimulated when nicked over 20 times with the *Hae*III enzyme. Yoshihara *et al.* (1977), by using enzyme-saturation studies, compared the ability of various DNA molecules to support poly(ADP-ribose) synthesis. Calculations showed maximal activity at approx. 200 base-pairs with highly polymerized calf thymus DNA, 40 base-pairs with poly(dA)·poly(dT) and only 10 base-pairs with an 'active' DNA recovered during purification of the enzyme. This active DNA has been partially characterized (Yoshihara *et al.*, 1978; Hashida *et al.*, 1979). It had a mol.wt. of 200000 and a GC content of 43%.

Histones have been reported by many groups (see, e.g., the references in Table 1) to stimulate the activity of purified poly(ADP-ribose) synthetase. Yoshihara *et al.* (1978) showed that although histones stimulated ADP-ribosylation 100 or 400% with intact or partially denatured calf thymus DNA respectively, activity was near maximal even without histones in the presence of poly(dA)·poly(dT) or 'active' DNA; stimulation by histones was only 8–25%. They suggest histones may stimulate

Table 1. *Purification of poly(ADP-ribose) synthetase*

Tissue	Purification (fold)	Yield (%)	Reference
Pig thymus	85	>100	Khan & Shall (1976)
	34	>100	Khan & Shall (1976)
	2500	50	Tsopanakis *et al.* (1976)
	7525	70	Tsopanakis *et al.* (1977)
	9235	46	Tsopanakis *et al.* (1978*a*)
Calf thymus	550	3.4	Okazaki *et al.* (1976)
	3000	6	Mandel *et al.* (1976)
	1300	10–20	Yoshihara *et al.* (1978)
	5000	15	Okayama *et al.* (1977)
	1250	14	Ito *et al.* (1979)
Ehrlich ascites cells	131	33	Kristensen & Holtlund (1976)
	700	25	Kristensen & Holtlund (1978)
Chronic-leucocytic-leukaemia cells	No data given		Ellison (1978)

synthesis by masking inhibitory sections of DNA. Okayama *et al.* (1977) showed that histones when added to purified enzyme and DNA were not ADP-ribosylated themselves, but activated synthesis in some other way. The same group (Ueda *et al.*, 1979) have since shown that an ADP-ribose–histone-H1 conjugate, synthesized by the Schiff-base method of Kun *et al.* (1976), was able to act as an acceptor in the same system. A possible explanation for this result is that 'initiating' enzymes, required for attaching the first 'ADP-ribose' moiety to protein, are either lost or inactivated during purification of the poly(ADP-ribose) synthetase. Yoshihara *et al.* (1977), however, reported that a purified enzyme preparation could incorporate ADP-ribose in the presence of DNA with the enzyme itself acting as acceptor. By performing incubations with NAD for different times, they found the mobility of the enzyme on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was altered. They also were able to show, together with Nolan (1978), by using pulse–chase experiments, that elongation of poly(ADP-ribose) occurred by addition of ADP-ribose on to the AMP terminus of the growing chain. Ueda *et al.* (1979) reached a similar conclusion after analysing the reaction product of incubations in the presence of ADP-ribose–histone-H1 conjugates.

Synthesis of poly(ADP-ribose) in isolated nuclei is inhibited by various groups of compounds. Substrate analogues such as α-NAD and $NADH_2$ are potent inhibitors (see Hilz & Stone, 1976). Poirier *et al.* (1978) reported that high concentrations of β-NAD also inhibited, suggesting negative co-operativity. End-product inhibition by nicotinamide and 5-methylnicotinamide has been demonstrated (eg. Clark *et al.*, 1971). The enzyme is also inhibited by thymidine and some of its analogues (Preiss *et al.*, 1971), although the significance of this inhibition is not understood. More recently, Levi *et al.* (1978) have shown that methylated xanthines and cytokinins were also good inhibitors. The most potent inhibitors are the 3-substituted benzamides (M. R. Purnell & W. J. D. Whish, unpublished work). The latter authors also showed 3-aminobenzoic acid and various acetophenones were good inhibitors of poly(ADP-ribose) synthetase.

Polyamines have been shown to stimulate ADP-ribosylation in isolated nuclei from various tissues (Muller & Zahn, 1976*a*; Tanigawa *et al.*, 1977; Perrella & Lea, 1978, 1979). A differential effect was reported in the presence and absence of Mg^{2+}. Non-histone proteins were predominantly modified in the presence of Mg^{2+}, whereas histones were predominantly modified in the absence of Mg^{2+} (Tanigawa *et al.*, 1977). Perrella & Lea (1978) showed that spermine caused increased ADP-ribosylation of histone H1 with concomitant decreased ADP-ribosylation of core histones. These effects cannot be explained by inhibition of degradative enzymes, since Whish's group (Whitby *et al.*, 1979) showed that polyamines caused no inhibition of poly(ADP-ribose) glycohydrolase in isolated nuclei from wheat seed. Polyamines appear to cause changes in ADP-ribosylation by altering the accessibility of acceptor proteins rather than by stimulating poly(ADP-ribose) synthetase (see section 2*h*).

Recently two groups have developed nucleotide-permeable cell systems with which to perform poly(ADP-ribose) synthetase measurements (Halldorsson *et al.*, 1978; Berger *et al.*, 1978*a*). These authors believe that such systems more closely reflect enzyme activity *in vivo* than do the isolated nuclei used previously. Halldorsson *et al.* (1978) showed permeabilized L-cells incorporate [^{3}H]ADP-ribose at a much lower rate than do isolated nuclei. Preincubation of permeabilized cells resulted in enzyme activity more closely resembling isolated nuclei. However, when the size of DNA was determined by alkaline sucrose density gradients, the higher activities appeared to be a result of the DNA damage. This finding was confirmed by preincubating permeable cells in the presence of the factors required for DNA repair. In this case no increase in activity was observed. Berger *et al.* (1978*a*) performed a much fuller characterization of permeable L-cells and the results obtained are reported in section (3). Perhaps most significant was the observation that added deoxyribonuclease greatly stimulated enzyme activity.

(*e*) *Degradation of poly(ADP-ribose).* Poly(ADP-ribose) has two bonds that are known to be susceptible to enzymic hydrolysis. The pyrophosphate bond is cleaved endonucleolytically by a phosphodiesterase from the snake *Crotalus adamanteus*, producing phosphoribosyl-AMP and 5′-AMP (Chambon *et al.*, 1966; Nishizuka *et al.*, 1967; Fujimura *et al.*, 1967). Exonucleolytic cleavage from the AMP terminus is mediated by a phosphodiesterase from rat liver (Futai *et al.*, 1967, 1968; Matsubara *et al.*, 1970). A phosphodiesterase, of unknown specificity, from cultured tobacco (*Nicotiana tabacum*) cells (Miwa *et al.*, 1975*a*; Shinsi *et al.*, 1976), also cleaves the polymer. However, deoxyribonuclease, ribonuclease, micrococcal nuclease, spleen phosphodiesterase and nucleotide pyrophosphatase have no effect on poly(ADP-ribose) (Hasegawa *et al.*, 1967).

The ribose–ribose bond is cleaved by an enzyme called poly(ADP-ribose) glycohydrolase. This enzyme was first discovered in the nuclei of calf thymus by Miwa & Sugimura (1971), who found it to be mainly associated with chromatin. It has been purified 200-fold with 5% yield and cleaves poly(ADP-ribose) exonucleolytically, leaving the terminal ADP-ribose moiety attached to protein. The enzyme was inhibited by cyclic AMP, ADP-ribose and *p*-chloromercuribenzoate (Miwa *et al.*, 1974). Ueda *et al.* (1972) have shown that poly(ADP-ribose) glycohydrolase occurs in a rat liver nuclei soluble fraction. This enzyme appears to be identical with enzyme found in the insoluble chromatin fraction (Miyakawa *et al.*, 1972). It has also been found in mouse L-cell nuclei (Stone *et al.*, 1973), the slime mould *Physarum polycephalum* (Tanaka *et al.*, 1976), in wheat embryo (Whitby & Whish, 1978) and in rat testis (Burzio *et al.*, 1976). A comparison of properties is found in Tanaka *et al.* (1976). On the basis of different pH optima and KCl stimulation there appear to be two enzymes present in rat testis. Miwa *et al.* (1975*b*) have shown that in various rat tissues poly(ADP-ribose) glycohydrolase is the major degradative enzyme for poly(ADP-ribose).

DNA has been shown to inhibit poly(ADP-ribose)glycohydrolase in several systems (see Hilz & Stone, 1976). Denatured DNA is an extremely potent inhibitor of the enzyme. Double-stranded DNA has a variable inhibitory effect that may simply be a reflection of the single-stranded-DNA impurities in the preparations used. Stone *et al.* (1978) showed that the enzyme was bound more tightly to denatured DNA–cellulose than to double-stranded-DNA–cellulose. Inhibition of the enzyme by high concentrations of DNA could be overcome by either increasing the ionic strength of the medium or by adding histone H1, and they suggested that both of these treatments may be displacing the enzyme from inhibitory (possibly single-stranded) portions of DNA.

Synthesis of histone H1 'dimer' (Stone *et al.*, 1977) in nuclei of various cell types showed an inverse correlation with poly(ADP-ribose) glycohydrolase activity (Lorimer *et al.*, 1977). Thus HeLa-cell nuclei producing histone H1 'dimer' possessed no detectable poly(ADP-ribose) glycohydrolase activity, whereas MTW-9 rat mammary nuclei had high enzyme activities but no dimer. It was shown that calf thymus poly(ADP-ribose) glycohydrolase (purified 200-fold by the method of Miwa *et al.*, 1975) hydrolysed histone H1 dimer from HeLa cells at one-ninth the rate of an equivalent mass of poly(ADP-ribose)$_{15}$. The rate of degradation of the 'dimer' was increased 2-fold by the addition of DNA at a ratio of 2 : 1 with histone H1 (Stone *et al.*, 1978), suggesting that DNA may cause a conformational change in the structure of the 'dimer'.

Various workers have observed that poly(ADP-ribose) glycohydrolase does not remove the final ADP-ribose residue from protein (Burzio *et al.*, 1976; Miwa *et al.*, 1974; Stone *et al.*, 1977). More recently, Okayama *et al.* (1978*b*) have shown the presence of an enzyme in rat liver nuclei that removes the ADP-

ribose from mono(ADP-ribosyl)ated histone H2B. The reaction product is not, however, ADP-ribose.

(2) *Biological role of ADP-ribosylation*

(*a*) *Introduction.* Since Hogeboom & Schneider (1952) discovered that NAD pyrophosphorylase is exclusively located in the nucleus, various authors have suggested that NAD fulfils a role in addition to that of respiratory coenzyme in eukaryotic cells. As early as 1958, Morton suggested that it may hold a key role in the regulation of cell proliferation in that the nucleus may sense changes in intracellular NAD concentrations. The discovery in 1966–1967 by three groups (Chambon *et al.*, 1966; Nishizuka *et al.*, 1967; Hasegawa *et al.*, 1967) that a nuclear enzyme, poly(ADP-ribose) synthetase, utilized NAD as substrate for the modification of nuclear proteins, suggested that such an enzyme may provide the mechanism by which NAD concentrations are sensed. That this enzyme plays an important physiological role is suggested by the surprisingly high turnover of NAD in the cell. In D98/AH2 cells, Rechsteiner *et al.* (1976) have shown that the half-life of NAD is 1 h. They had previously shown that, in cells enucleated by cytochalasin, the half-life of NAD was 10 h (Rechsteiner *et al.*, 1974). From these data they suggested that poly(ADP-ribose) synthetase was the major degradative enzyme of NAD. The magnitude of the turnover of NAD can best be expressed by the fact that more adenine leaves NAD than enters DNA.

Since the discovery of poly(ADP-ribose) synthetase, however, its biological role remains unclear. The earliest approach to determine its function was to isolate nuclei from cells in various states and attempt to correlate the activity of the enzyme with a particular cell state. As stated previously (section 1*d*) the validity of enzyme-activity measurements from isolated nuclei must be treated with caution. The permeabilized-cell system might provide a more realistic model for the study of enzyme activity than the use of isolated nuclei. Another approach has been to correlate the concentrations of ADP-ribosylated proteins *in vivo* with a particular cell status. The difficulties in measuring ADP-ribosylation of proteins *in vivo* have been mentioned above. In addition no methods have been devised to look at changes in the protein species modified.

Another approach to elucidating the role of the ADP-ribosylation has been the use of inhibitors of poly(ADP-ribose) synthetase *in vivo*. The use of nicotinamide, 5-methylnicotinamide, thymidine and methylated xanthines as physiological inhibitors must be treated with reservation, since all are known to affect cellular processes other than ADP-ribosylation. It is considered that the use of 3-aminobenzamide and especially 3-methoxybenzamide will be extremely useful in this context.

A major problem in ascribing a precise role for ADP-ribosylation has been the difficulty in correlating the data obtained by the above methods with a defined cell function; e.g., during differentiation of a particular cell, the rate of DNA synthesis will alter, the patterns of DNA transcription will change and the cell may enter a different stage of the cell cycle.

For these and other reasons, attempts to elucidate the biological role of ADP-ribosylation have produced wide-ranging and contradictory suggestions for its function. Therefore, with these reservations in mind, the various cellular functions that have been ascribed to poly(ADP-ribose) are presented below.

(*b*) *DNA synthesis and cellular proliferation.* Since 1970, when Burzio & Koide showed that preincubation of isolated rat liver nuclei with NAD caused a decrease in incorporation of [^{3}H]dTTP into acid-insoluble material, many workers have studied the possible involvement of ADP-ribosylation in the regulation of DNA synthesis. The results obtained show that ADP-ribosylation in isolated nuclei either decreases, has no effect or increases dTTP incorporation into DNA [for a summary, see Hilz & Stone (1976) and Hayaishi & Ueda (1977)].

Since that time, Janakidevi (1978) has shown that removal of lysine-rich histones or treatment with heparin increases DNA synthesis in isolated nuclei from pig aorta. The decrease in poly(ADP-ribose) synthesis observed with lysine-rich histone is explained by co-extraction of poly(ADP-ribose) synthetase, and they conclude that removal of lysine-rich histones or poly(ADP-ribose) synthetase exposes initiation sites for DNA synthesis. Tanigawa's group (see references below) have shown that preincubation of isolated nuclei from chick-embryo liver with NAD increased dTTP incorporation into DNA. Conversely, preincubation with NAD of nuclei from hen liver decreased dTTP incorporation. Extraction of NAD-treated embryonic and adult liver nuclei with 0.35 M-NaCl with subsequent reconstitution showed that the factors responsible for suppression or stimulation of DNA synthesis were present in the 0.35 M-NaCl extract (Tanigawa *et al.*, 1978*a*). They later showed that the stimulation of DNA synthesis observed with chick-embryo nuclei was due to increased accessibility of the DNA to nuclease (Tanigawa *et al.*, 1978*b*). Administration of glucocorticoid hormone to chick embryos caused decreases in both DNA and poly(ADP-ribose) synthesis (Kitamura *et al.*, 1979). This may be due to loss of enzyme activity as a result of increased nuclear fragility.

Miwa *et al.* (1977*a*) showed that poly(ADP-ribose) synthetase activity was 2–10-fold higher in nuclei from SV40-virus-transformed cells compared with untransformed cells. They also demonstrated that, in contrast with untransformed cells (where enzyme activity remained constant), the activity in transformed cells increased markedly throughout the growth cycle. Muller *et al.* (1979) found that alteration of DNA synthesis by HS (herpes simplex)-virus infection was not accompanied by a change in poly(ADP-ribose) synthetase activity in BHK cells, suggesting that ADP ribosylation plays no role in the control mechanisms for cellular or HS-virus DNA synthesis.

Ghani & Hollenberg (1978*a*) demonstrated that chick embryo heart cells exhibited higher poly(ADP-ribose) synthetase activity in isolated nuclei from cells grown in 5% (v/v) O_2 than from cells grown in 20% O_2. They later showed, by pulse-labelling the cells with adenosine, an increased amount of poly(ADP-ribose) *in vivo* in cells grown in 20% O_2. They suggested that in rapidly dividing cells (5% O_2) the redox potential shifts ($NAD^+ \rightarrow NADH$), causing a decrease in poly(ADP-ribose) synthesis and thus increased DNA synthesis (Ghani & Hollenberg, 1978*b*).

Suhadolnik *et al.* (1977) made an interesting observation when studying the effect of various NAD analogues on dTTP incorporation into isolated nuclei from foetal-rat liver. When preincubated with 0.5 mM-NAD^+, only 9% inhibition of dTTP incorporation was observed. However, 2′-deoxy- and 3′-deoxy-NAD both caused 90% inhibition. The significance of this phenomenon was not explained.

From previous data and the results shown above, serious doubts must be raised as to the suitability of isolated nuclei as physiological models for ADP-ribosylation and semi-conservative DNA synthesis. Berger *et al.* (1978*a*) have suggested that permeabilized cells are better models for studying ADP-ribosylation than isolated nuclei, and have also found that incorporation of [^{3}H]dTTP into acid-insoluble material does represent semi-conservative DNA replication (Berger & Johnson, 1976). The results obtained with this system are described below. Berger *et al.* (1978*a*) also showed that inhibition of poly(ADP-ribose) synthetase had no effect on DNA synthesis, and that inhibitors of DNA synthesis had no effect on poly(ADP-ribose) synthesis in permeabilized L-cells. They also demonstrated that when simultaneous synthesis of both polymers occurred, the synthesis of one did not affect the other. Furthermore, it was found that the rate of synthesis of poly(ADP-ribose) was greater in stationary-phase cells than in exponential-phase cells, whereas DNA synthesis was higher in the exponential phase. However, the total poly(ADP-ribose) synthetase activity did not vary during the growth cycle when the enzyme was measured in the presence of deoxyribonuclease I and Triton X-100. Similarly

they were able to show that decreased DNA synthesis in cells subjected to acute glucose deficiency, vaccinia virus infection and cytosine arabinoside treatment, resulted in increased poly(ADP-ribose) synthetase activity in permeabilized cells (Berger *et al.*, 1978*a*,*b*).

When normal and chronic lymphocytic leukaemia (CLL) lymphocytes were examined with respect to phytohaemagglutinin stimulation (Berger *et al.*, 1978*d*), differences were observed. In both normal and CLL lymphocytes phytohaemagglutinin stimulation caused increased ADP-ribosylation. The normal cells showed the expected response of DNA synthesis to phytohaemagglutinin stimulation, but the response of DNA synthesis in the CLL cells was decreased and much delayed. The authors suggested that this may be due to either damaged or disordered DNA (section 1*d*) or the presence of immature differentiating lymphocytes.

Finally, Hilz's group (R. Bredehorst, M. Goebel, F. Renzi, M. Kittler, K. Klapproth & H. Hilz, unpublished work) have examined the activity of poly(ADP-ribose) synthetase in permeabilized cells and the concentrations *in vivo* of mono(ADP-ribosyl)ated proteins throughout the growth cycle of Ehrlich ascites-tumour cells. They showed that transition from exponential to stationary phase was associated with an increase in both intrinsic poly(ADP-ribose) synthetase activity and hydroxylamine-resistant mono(ADP-ribosyl)ated proteins. The total poly(ADP-ribose) synthetase activity and hydroxylamine-sensitive mono(ADP-ribosyl)ated proteins remained unchanged.

Attempts have been made to provide a more realistic model for the role of ADP-ribosylation in DNA synthesis by incubating tissue slices with NAD^+ (Claycomb, 1976*a*). However, results from such experiments are impossible to interpret, since NAD^+ does not cross the cell membrane. It is noteworthy that NAD^+ stimulates DNA synthesis in human bone-marrow cells. The authors suggested that this may be due to an exogeneous enzyme system (Schacter & Burke, 1978).

(*c*) *DNA transcription.* The observation that differentiated rat liver cells engaged in RNA synthesis possessed a higher activity of poly(ADP-ribose) synthetase than cells engaged primarily in DNA synthesis (Haines *et al.*, 1969) was the first observation suggesting a possible role for ADP-ribosylation in DNA transcription. Since then, several reports have emerged that agree or conflict with such a role for ADP-ribosylation. One approach that has been made to clarify this situation has been to correlate poly(ADP-ribose) synthetase activity with the RNA-synthesizing capacity in various systems. Thus it was shown that there was no change in poly(ADP-ribose) synthetase activity in livers from sham-operated, adrenalectomized and cortisol-treated rats (Hilz & Kittler, 1971), suggesting no role in DNA transcription. Also the observation that the specific activity of poly(ADP-ribose) synthetase in nuclei and nucleoli isolated from *Tetrahymena* is the same, despite the high ribosomal transcriptional activity in the nucleoli, suggests no direct involvement of ADP-ribosylation in transcription. Furthermore, in the presence of active RNA synthesis, the poly(ADP-ribose) synthetase activity in both nuclei and nucleoli was the same as in the absence of RNA synthesis (Tsopanakis *et al.*, 1978*b*). The involvement of ADP-ribosylation in transcription has been implicated from the results of Muller *et al.* (1974). These workers made the observation that, during gene expression *in vivo* initiated by oestrogen treatment of immature quails, an increase in RNA polymerases I and II activities is accompanied by a decrease in poly(ADP-ribose) synthetase activity in the oviduct. A positive association of poly(ADP-ribose) synthetase activity with transcription has been suggested from the studies of Smulson and co-workers, who have fractionated sonicated HeLa-cell chromatin by using ECTHAM-cellulose chromatography and glycerol-gradient centrifugation in an attempt to separate transcriptionally active and inactive chromatin (Mullins *et al.*, 1977). These authors concluded that the poly(ADP-ribose) synthetase activity is primarily associated with extended transcriptionally active chromatin; the transcriptionally inactive, condensed, chromatin fractions contained relatively low poly(ADP-ribose) synthetase activity. This conclusion has been questioned by Yukioka *et al.* (1978), who have pointed out that the chromatin-fractionation technique using mechanical shearing, as used by Mullins *et al.* (1977), is not adequate for the separation of transcriptionally active and inactive chromatin fractions and also that sonication causes drastic changes in chromatin structure [see Yukioka *et al.* (1978) for chromatin-preparation references]. Rat liver chromatin has been fractionated by using selective shearing with deoxyribonuclease II followed by preferential precipitation, which has been shown to fractionate chromatin into transcriptionally active and inactive fractions (Gottesfeld *et al.*, 1974, 1975), and it was shown that poly(ADP-ribose) synthetase activity is not preferentially localized in transcriptionally active chromatin regions (Yukioka *et al.*, 1978).

A second approach has been to look at the effect of poly(ADP-ribosyl)ation in isolated nuclei on subsequent RNA transcription. Thus formation of poly(ADP-ribose) in isolated rat liver nuclei did not affect the subsequent RNA synthesis by the nuclei (Burzio & Koide, 1971). Muller *et al.* (1974) have shown that, after preincubation of quail oviduct nuclei with NAD^+, the activity of endogenous RNA polymerase I decreased, whereas the activity of RNA polymerase II as well as exogenous bacterial RNA polymerase remained unaffected by the ADP-ribosylation reaction. The data was suggestive of a direct ADP-ribosylation of the RNA polymerase I. Furthermore, the amount of poly(ADP-ribose) associated with RNA polymerase I isolated from nuclei of oviducts undergoing increased transcription was seen to be lower than that associated with the RNA polymerase I from nuclei of oviducts with moderate transcription (Muller & Zahn, 1976*b*). More recently it has been demonstrated that preincubation of BHK cell nuclei with NAD^+ resulted in an inhibition of RNA polymerase I, and it was suggested that the inhibition was due to ADP-ribosylation of the RNA-polymerase itself, as evidenced by an inhibition of RNA polymerase activity when extracted and assayed from nuclei previously incubated with NAD^+ (Furneaux & Pearson, 1978).

It has been shown that protein A24, which is composed of histone H2A and ubiquitin, a non-histone protein linked by an isopeptide bond (Goldknopf & Busch, 1977) is ADP-ribosylated in isolated rat liver nuclei (Okayama & Hayaishi, 1978). In view of the possible role of protein A24 as a repressor of ribosomal gene activity (Ballal & Busch, 1973; Ballal *et al.*, 1974, 1975), it has been suggested that ADP-ribosylation of this protein may be involved in the regulation of its repressor activity (Okayama & Hayaishi, 1978).

(*d*) *DNA repair.* Studies on a possible involvement of ADP-ribosylation in DNA repair have been based on two observations. First, it has long been known that alkylating agents and other treatments known to damage DNA result in depletion of intracellular NAD^+ (Roitt, 1956) and, secondly, that damaged DNA stimulates the activity of poly(ADP-ribose) synthetase (see section 1*d*).

It was shown by Smulson *et al.* (1975) and Whish *et al.* (1975) that isolated nuclei, from HeLa cells and the slime-mould *Physarum polycephalum* respectively, possessed higher poly(ADP-ribose) synthetase activity after pretreatment with streptozotocin, the 2-deoxy-D-glucose derivative of the alkylating agent, *N*-methyl-*N*-nitrosourea. Jacobson (1978) examined the effect, on NAD^+ concentrations, of *N*-nitroso compounds that were direct-acting, indirect-acting or non-carcinogens in both 3T3 cells and mitogen-stimulated human lymphocytes. They observed large decreases in NAD^+, and in both cell types treated with direct-acting carcinogens, a large decrease in lymphocytes, but not in 3T3 cells treated with indirect-acting carcinogens, whereas non-carcinogens had no effect on NAD^+ concentrations in either cell type.

Sudhakar *et al.* (1979*a*) showed that the *N*-methyl-*N*-nitro-

sourea-induced increase in poly(ADP-ribose) synthetase at the nucleosome level was a result of increased availability of protein acceptors. In a subsequent paper (Sukhakar *et al.*, 1979*b*), they compared alkylation of chromatin by *N*-methyl-*N*-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. In contrast with *N*-methyl-*N*-nitrosourea, treatment of HeLa cells with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea resulted in a slight decrease in poly(ADP-ribose) synthetase activity in isolated nuclei. They were able to show, by nuclease digestion, that the differential effects of the two compounds on poly(ADP-ribose) synthetase may be explained by the sites of alkylation of the two compounds. *N*-methyl-*N*-nitrosourea preferentially alkylates the internucleosomal regions of DNA, which have been suggested as the sites of the enzyme binding (Mullins *et al.*, 1978), whereas 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea alkylates the core particles.

Other treatments known to damage DNA have also been shown to stimulate poly(ADP-ribose) synthetase activity in isolated nuclei. Davies *et al.* (1976, 1977) have demonstrated increased enzyme activity in cells treated with γ-irradiation and with the polypeptide antitumour antibiotic neocarzinostatin.

Berger *et al.* (1979) have demonstrated increased poly(ADP-ribose) synthesis in permeabilized cells pretreated with u.v. irradiation. They were further able to show that the DNA synthesis observed after irradiation consisted of repair synthesis and not semi-conservative replication.

An interesting observation is that compounds such as theophylline and caffeine, known inhibitors of DNA repair (Cleaver & Thomas, 1969; Lehmann & Kirk-Bell, 1972), are also inhibitors of poly(ADP-ribose) synthetase in isolated nuclei (Levi *et al.*, 1978). It was therefore of interest to examine the effect of other inhibitors of poly(ADP-ribose) synthetase on the ability of cells to repair DNA damage *in vivo* and their subsequent survival. The earliest study was performed by Smulson *et al.* (1977). At 2 days after injection of mice with LS1210 tumour cells, *N*-methyl-*N*-nitrosourea was injected in the presence and absence of nicotinamide. Mice injected with *N*-methyl-*N*-nitrosourea showed increased survival over controls, and co-administration of nicotinamide slightly increased survival over animals treated with *N*-methyl-*N*-nitrosourea alone. Shall *et al.* (1977) showed that co-administration of doses of 5-methylnicotinamide or theophylline, sufficient to prevent streptozotiocin-induced decreases in NAD^+ in L-cells, increased the cytotoxicity of streptozotocin, although they were not cytotoxic in themselves.

Finally, it has been shown that 3T3 cells depleted of NAD^+ by culture in nicotinamide-free medium were unable to undergo carcinogen-induced unscheduled DNA synthesis (Jacobson & Narasimhan, 1979). All these results suggest that ADP-ribosylation of nuclear proteins plays some, as yet unknown, role in the repair of DNA.

(*e*) *Cell cycle.* Many workers have investigated the possible involvement of ADP-ribosylation of nuclear proteins in the timing of events during the cell cycle. Before 1976, all studies were performed by isolating nuclei from synchronized cells and determining the enzyme activity. Although no consistent picture emerged, most results suggested that poly(ADP-ribose) synthetase activity was lowest in S-phase and highest in either G_2- or G_1-phase (Hilz & Stone, 1976). Tanuma *et al.* (1978) used intact and disrupted nuclei and a crude poly(ADP-ribose) synthetase preparation to measure the activity of the enzyme during the cell cycle of HeLa S3 cells. They showed that the activity of the enzyme in isolated nuclei was higher in the G_2-phase, whereas in disrupted nuclei the activity increased during G_1-phase, remained elevated during G_2-phase and decreased after mitosis. By using crude enzyme they found a pattern similar to that obtained with disrupted nuclei. By using colcemid, a mitotic inhibitor, they found that mitotic cells had five times the activity of asynchronous cells as measured by the isolated-nuclei system. From this and previous data it is clear that isolated nuclei are unsuitable for elucidating the role of ADP-ribosylation during the cell cycle.

Berger *et al.* (1978*b*) showed that, after treatment of LS1210 cells with cytosine arabinoside (which causes accumulation of cells in G_2-phase), the activity of poly(ADP-ribose) synthetase increased when measured in permeabilized cells. By using Chinese-hamster ovary cells synchronized by mitotic selection they showed that the activity of the enzyme increased during G_1-phase, decreased rapidly as the cells traversed S-phase and increased during G_2-, M- and G_1-phases (Berger *et al.*, 1978*c*). By measuring the activity in permeabilized cells after deoxyribonuclease treatment, which was suggested to indicate total enzyme activity, they showed the activity was constant during the cell cycle except for a small peak during late S-phase, which subsequently decreases during the G_2- and M-phases. They suggested that the enzyme was synthesized during S-phase and then returned to normal amounts after mitosis. This observation may account for the maxima observed during S-phase observed by other workers using isolated nuclei (Roberts *et al.*, 1973; Colyer *et al.*, 1973).

Kidwell & Mage (1976) used a radioimmunoassay to detect changes in poly(ADP-ribose) concentrations in HeLa cells during the cell cycle. They observed a 6-fold increase in poly(ADP-ribose) as the cells traversed from early S- to late S-phase, followed by a rapid decrease. This was followed by a 10-fold increase during G_2-phase; this increase was correlated with increased poly(ADP-ribose) synthetase activity as measured in isolated nuclei. As the authors state, more information is required on the specificity of the antibody used.

Hilz's group (Wielckens *et al.*, 1979) used an antibody to 5′-AMP to measure hydroxylamine-sensitive and -resistant mono-(ADP-ribose)–protein conjugates during the cell cycle of the naturally synchronous slime mould *Physarum polycephalum*. They observed that total mono(ADP-ribose) decreased from mitosis through S-phase (G_1-phase is absent) and increased through G_2- to M-phase. When the system was analysed for hydroxylamine-lability they found different patterns for hydroxylamine-sensitive and -resistant mono(ADP-ribose)–proteins. Amounts of hydroxylamine-sensitive residues decreased sharply after mitosis, remained low during S-phase and increased at the S-phase/G_2-phase boundary and then remained at that value until mid-G_2-phase; they then decreased during late G_2-phase and rapidly increased before mitosis. Amounts of hydroxylamine-resistant residues decreased after mitosis but increased linearly during S-phase; from the S-phase/G_2-phase boundary they remained constant until mid-G_2-phase and then increased to pre-mitotic values. These data suggest ADP-ribosylation may fulfil more than one role during the cell cycle.

Caplan *et al.* (1978) used partially hepatectomized rat liver to investigate changes in poly(ADP-ribosyl)ation in isolated nuclei after transition from G_0- to S-phase. They could detect a slight increase in ADP-ribosylation but could detect no change in the proteins released after digestions with nuclease. Since synchrony is only 30%, the methods may not be sufficiently sensitive to detect changes.

(*f*) *Cellular differentiation and development.* Caplan & Rosenberg (1975) looked at the differentiation of mesodermal cells of embryonic-chick limb buds into either muscle or cartilage, and their results were the first to suggest a possible involvement of ADP-ribosylation in differentiation. Previous work from Caplan's laboratory had shown a correlation between pyridine nucleotide concentrations and cellular differentiation, and they suggested that fluctuations in cellular NAD^+ concentrations might play a role in the control of muscle as against cartilage development [see Caplan & Rosenberg (1975) for references]. The authors showed that 3-acetylpyridine potentiated cartilage differentiation that was associated with a stimulation of the rate of poly(ADP-ribose) synthesis. Administration of 3-acetylpyridine had previously been shown to decrease NAD concentrations, and the suggested mechanism was that such a change in

NAD^+ concentration is 'sensed' and transposed into differential rates of ADP-ribosylation that are correlated with differentiation into either muscle or cartilage (Caplan & Rosenberg, 1975). This proposed role of 3-acetylpyridine in repressing myogenesis and enhancing chondrogenesis, however, has been questioned by McLachlan *et al.* (1976), who made an histological analysis of developing limb buds at various stages. They showed that the effect of 3-acetylpyridine was mediated via a destruction of the peripheral nerves, resulting in a total loss at 24h, as well as a deleterious effect on all cell types, including cartilage, tendon, mesenchyma and muscle. Such results suggest that 3-acetylpyridine affects muscle growth rather than muscle differentiation, as does the observation that 3-acetylpyridine still has an effect on muscle tissue at stages when cartilage and muscle cells are physically separated (McLachlan *et al.*, 1976).

Since the first observation of Caplan & Rosenberg (1975), several reports have appeared that may suggest an involvement of poly(ADP-ribose) in differentiation. For example, during the early stages of oocyte maturation in the toad *Xenopus*, which is mediated by progesterone, the oocyte nucleus breaks down and this is followed by a dramatic condensation of the chromosomes. It has been shown that, during such oocyte maturation, poly(ADP-ribose) synthetase activity in isolated nuclei increases 3-fold, this dramatic increase occurring before breakdown of the oocyte nucleus (Burzio & Koide, 1977). Such results suggest a possible role for ADP-ribosylation in the early differentiation of *Xenopus*, and the observation (Farzaneh & Pearson, 1977) that the activity of poly(ADP-ribose) synthetase increases markedly during its embryonic development is consistent with this possibility.

Young & Sweeney (1978) have studied the incorporation of [^{3}H]adenosine into RNA and ADP-ribosylated protein in mouse ova and have shown that the total incorporation is maximal at 3–5h after ovulation, which is the time of normal fertilization. The incorporation of [^{3}H]adenosine is low at 7–8h, at which time the ability of the ovum to be fertilized is decreased; this suggests a possible role between adenosine metabolism, mediated in part through poly(ADP-ribosyl)ation, and post-ovulation maturation of ova and/or their fertilizability. The above authors have also shown that mouse one-cell embryos incorporate adenosine into ADP-ribosylated material and they found that the average chain length of the polymer was 2 (a dimer) (Young & Sweeney, 1979) compared with the pentamer and monomer seen in the ovum (Young & Sweeney, 1978). In view of the apparent difference in lability of the bond linking the dimer to its acceptor in the embryo compared with the pentamer and monomer in the ovum, it was proposed that the dimer was synthesized after fertilization (Young & Sweeney, 1979). Such differential ADP-ribosylation in the ovum and embryo may indicate that a differentiation-related programme of poly(ADP-ribose) synthesis may be taking place in this system.

It has been shown by Yamada *et al.* (1978) that mouse myeloid leukaemic cells can be induced to differentiate into cells with phagocytic activity, Fc receptors and lysozyme activity, by the addition of poly(ADP-ribose) to the culture medium and that a certain proportion of these cells differentiate further into granulocytes and macrophages. Addition of dextran sulphate or poly(vinyl sulphate) was also effective in the induction of phagocytic cells and so the physiological significance of the observed stimulation of differentiation by poly(ADP-ribose) must remain questionable, although the radioactivity derived from polymer does enter the cells and becomes located in the nucleus and nuclear membrane as seen by radioautography.

Two reports have appeared that have investigated the possible role of poly(ADP-ribosyl)ation in the differentiation of erythroleukaemic mouse spleen cells (Friend cells) grown in culture. Rastl & Swetly (1978), using cell line F4N, which is reported to be very sensitive to butyrate-induced differentiation and relatively insensitive to hexamethylenebisacetamide (Morioka *et al.*, 1979), showed that using such compounds to induce differentiation resulted in a 2–4-fold increase in poly(ADP-ribose) synthetase activity as well as a concomitant transition of cells to the G_1-phase of the cell cycle. Morioka *et al.* (1979), using cell line 745, showed that poly(ADP-ribose) synthesis suppression is detectable in the early-exponential-phase growth stage (14–24h) after treatment with hexamethylenebisacetamide or dimethyl sulphoxide. With butyrate, a transient increase in ADP-ribosylation in the early stages of cell growth was seen, but by 48–72h ADP-ribosylation was at the same low rate as for hexamethylenebisacetamide and dimethyl sulphoxide. Nicotinamide, too, induced differentiation and also enhanced dimethyl sulphoxide- and hexamethylenebisacetamide-induced differentiation but tended to inhibit butyrate-induced differentiation. Friend-cell variants unresponsive to hexamethylenebisacetamide and dimethyl sulphoxide did not exhibit as low an activity of poly(ADP-ribose) synthetase as was found with normal responsive cells treated with inducers. The effect of the inducers was not mediated by a change in cell growth rate or by an effect on polymer degradation, and the results suggest that the amount of ADP-ribosylation is correlated with the differentiation of the Friend cell (Morioka *et al.*, 1979). The reason for this discrepancy between the observations of Morioka *et al.* (1979) and Rastl & Swetly (1978) remains to be solved, but it could be related to differences in the strain of cells used and/or differences in the culture methods. For example, Morioka *et al.* (1979) cultured their cells in the presence of inducers without medium change, whereas Rastl & Swetly (1978) added fresh medium to their cultures daily. Indeed Rastl & Swetly (1978) did show that the amount of poly(ADP-ribose) synthesis depended on the culture conditions.

The activity of poly(ADP-ribose) synthetase in isolated nuclei and the intracellular NAD^+ concentration have been measured in differentiating rat cardiac muscle. NAD^+ concentrations increase during post-natal development as does poly(ADP-ribose) synthetase activity (Claycomb, 1976*b*). It was suggested that the initial increase in these two parameters may be specifically related to the 30–40-fold decrease in DNA synthesis observed during this initial period and that the further increase after the second week may be concerned with terminal cell differentiation.

(*g*) *Other possible functions of ADP-ribosylation.* In addition to the above-stated postulated roles, three other possible functions have been suggested:

(i) The observation that nicotinamide, 5-methylnicotinamide, thymidine and 3-isobutyl-1-methylxanthine induce ornithine decarboxylase (EC 4.1.1.17) activity prompted Minaga *et al.* (1978) to suggest that ADP-ribosylation may be involved in the regulation of this enzyme. This is an intriguing observation, since the product of ornithine decarboxylase, spermine, has been shown to stimulate poly(ADP-ribose) synthetase in isolated nuclei (see section 1*d*). Recently, Hilz & coworkers (unpublished work have shown an inverse correlation between ornithine decarboxylase and poly(ADP-ribose) synthetase activities as measured by the permeabilized-cell system during the growth cycle of Ehrlich ascites tumour cells.

(ii) Suzuki & Murachi (1978) have shown that a nucleic acid-like inhibitor co-extracts with a chromatin-bound neutral proteinase from rat peritoneal macrophages. They found that inhibition of proteinase activity could be removed by preincubating the de-proteinized inhibitor fraction with a crude poly(ADP-ribose) glycohydrolase preparation from rat liver before addition to the assay mixture. Deoxyribonuclease 1, P1 nuclease and snake-venom phosphodiesterase has no effect. Thus they suggested that the inhibitor was similar to, but not identical with, poly(ADP-ribose). It was also shown that poly(ADP-ribose) with an average chain length of 30 residues was inhibitory.

(iii) Matinyan & Umanskii (1978) have shown that the 'polypeptide synthetase' activity of rat liver chromatin was enhanced by preincubation with NAD^+. This enhancement was inhibited

by the presence of nicotinamide or thymidine. On storage of chromatin for 20h at 4°C, a complete loss of polypeptide synthetase activity was observed. This, however, could be prevented by the presence of 3′:5′-cyclic AMP, an inhibitor of poly(ADP-ribose) glycohydrolase. They suggest that poly(ADP-ribose) may serve as an energy source for amino acid activation.

(*h*) *Mechanisms by which ADP-ribosylation may affect cellular processes.* The possible mechanisms by which ADP-ribosylation may affect cellular function are:

(i) Direct modulation of enzyme activity as proposed by Yoshihara *et al.* (1975) and Muller & Zahn (1976*b*) for ($Ca^{2+} + Mg^{2+}$)-dependent endonuclease and RNA polymerase respectively.

(ii) Modification of a regulatory protein such as protein A24 (Okayama & Hayaishi, 1978).

(iii) Alteration of chromatin structure.

Investigation of the first two possibilities awaits the identification and elucidation of the biological role of these proteins ADP-ribosylated *in vivo*. Mediation by changes in chromatin structure could occur at two levels. Firstly, it may allow enzymes, such as DNA-repairing enzymes, access to previously shielded DNA. The second possible role may be related to altering gross chromatin structure, such as occurs during chromosome condensation before mitosis. The very low concentration *in vivo* (see section 1*c*) would seem to preclude involvement of poly(ADP-ribose) as a major structural element, but may indicate that it acts as a signal to other enzymes or proteins. Data attempting to correlate poly(ADP-ribose) synthesis and chromatin structures are presented below.

It is well known that the basic structural unit of chromatin, the 'nucleosome', is composed of 140 base-pairs of DNA wound around an octamer of core histones (H2B, H2A, H3 and H4). Such core particles are joined by a 'linker' region of DNA, which is the site of histone H1 attachment [for a review on chromatin structure, see Kornberg (1977)]. The extremely low ADP-ribosylation of the core histones (see sections 1*b* and 1*c*) indicates that such modification has no role in maintaining the nucleosome structure.

Smulson and his colleagues (Mullins *et al.*, 1977; Giri *et al.*, 1978*b*) have shown that the poly(ADP-ribose) synthetase in HeLa-cell chromatin is located in the linker DNA region between adjacent nucleosomes. The enzyme activity was shown not to coincide with the position of core particles on sucrose gradients, but was present at a position enriched in mononucleosomes possessing linker regions (Giri *et al.*, 1978*b*). Polyacrylamide-gel analysis after separation of mono- and di-nucleosomes also showed that dimers and monomers containing linker regions possessed poly(ADP-ribose) synthetase activity (Giri *et al.*, 1978*b*).

The modification of nuclear proteins in nuclei and chromatin prepared from mid-S-phase HeLa cells has been investigated by Jump *et al.* (1979). Nuclease digestion of S-phase nuclei resulted in the release of nucleosomes enriched in ADP-ribosylated proteins and poly(ADP-ribose) synthetase activity as well as nascent DNA from the DNA replicating fork. The results show that the poly(ADP-ribose) synthetase activity is correlated with extended forms of chromatin undergoing DNA replication or repair (Jump *et al.*, 1979).

Besides determining the probable localization of the poly(ADP-ribose) synthetase activity within chromatin, Smulson and co-workers (Mullins *et al.*, 1977; Giri *et al.*, 1978*a*,*b*) have also looked at the proteins that are ADP-ribosylated both in nuclei and chromatin. When HeLa nuclei were incubated with NAD^+ before nuclease digestion and subsequent nucleosome analysis it was seen that ADP-ribosylated histones were preferentially associated with mono- and di-nucleosomes, whereas higher oligonucleosomes exhibited more extensive modification of non-histone proteins. The authors suggested that ADP-ribosylation of histones either occurs in nucleosomes that are more susceptible to nuclease or renders them more susceptible (Giri *et al.*, 1978*a*). Also, when the ADP-ribosylated proteins were analysed in whole nuclei or in various classes of nucleosomes, it was observed that in nuclei, histones H1 and H2B were the major acceptors, with histones H2A, H3, HMG protein and M1 and M4 proteins being modified to a lesser extent. However, with mono-, di- and tri-nucleosomes, very little histone modification occurred, except on histones H1 and H3.1, whereas ADP-ribosylation of HMG protein and M1 and M4 proteins was greatly enhanced. Thus it seems that the core histones are only modified when the chromatin is in a native conformation (i.e., in nuclei), and these results emphasize the importance of native chromatin structure in allowing the linker-located enzyme to interact with and modify core histones (Giri *et al.*, 1978*a*). This native or higher-ordered chromatin structure and its relationship to poly(ADP-ribosyl)ation has been further investigated in Smulson's laboratory (Butt *et al.*, 1978, 1979; Giri *et al.*, 1978*a*). The specific activity of the poly(ADP-ribose) synthetase rises to a maximum with chromatin of 8–10 nucleosomes in length and as the complexity of the chromatin increases with respect to nucleosome number, a fall and levelling off of specific activity occurs suggesting that a defined compact structure within the chromatin may limit the reaction (Butt *et al.*, 1978). Such a definite structural organization has been demonstrated by Finch & Klug (1976), who showed that nucleosomes can be organized into solenoids with approx. 6–9 nucleosomes per helical turn of the solenoid. A unique structure of this periodicity has recently been demonstrated for HeLa-cell chromatin (Butt *et al.*, 1979) and for rat liver chromatin (Stratling *et al.*, 1978), both observations showing preferential cleavage of chromatin by micrococcal nuclease at periodicity of 8 and 16 nucleosomes. The higher specific activity of poly(ADP-ribose) synthetase in the octanucleosomes has been partially explained by the observation that more than 90% of the total incorporation in such structures occurred on protein C, which has a mol.wt. of 125000. Analysis of nucleosomes less than nine units in length showed the same pattern as octanucleosomes with progressively less protein C modification (Butt *et al.*, 1979). The suggestion has been made that protein C may be the poly(ADP-ribose) synthetase (Butt *et al.*, 1979; Jump *et al.*, 1979), and it has been speculated that the enzyme might be bound to chromatin at a periodicity of eight nucleosomes or in the mid-region of a 16-nucleosome structure (Butt *et al.*, 1979).

The poly(ADP-ribose) modification of chromatin proteins may function by influencing higher-ordered chromatin structure for the synthesis of DNA during replication or repair (Jump *et al.*, 1979). In view of the importance of histone H1 in maintaining higher-ordered chromatin structure (Finch & Klug, 1976; Renz *et al.*, 1977; Thoma & Koller, 1977; Worcel & Benyajati, 1977), an interesting possibility is that ADP-ribosylation of histone H1 may influence chromatin structure, in part, by causing cross-linking of histone H1 molecules located on non-adjacent linker regions within the chromatin. A dimer complex of histone H1 comprising two histone H1 molecules in association with a single chain of poly(ADP-ribose) has been shown to be synthesized in HeLa-cell nuclei (Stone *et al.*, 1977). Also, by using soluble chromatin preparations from HeLa-cell nuclei, Kidwell and his colleagues (Byrne *et al.*, 1978) have shown a correlation between induced chromatin condensation and the extent of histone H1–polymer complex synthesis. Consistent with this observation are the results of Perella & Lea (1978, 1979), which show that, in rat liver nuclei polyamines cause an increase in histone H1 ADP-ribosylation (and possibly histone H1–polymer dimer synthesis), which is accompanied by a decrease in ADP-ribosylation of the core histones. The speculation has been made that the formation of the histone–H1–poly(ADP-ribose) dimer complex may provide a mechanism for transiently condensing or stabilizing folds of chromatin fibres (Stone *et al.*, 1977; Lorimer *et al.*, 1977; Byrne *et al.*, 1978). The transient nature of the process has been inferred from the observation that the histone H1–polymer complex accumu-

lation is inversely related to the poly(ADP-ribose) glycohydrolase activity in the nuclear synthesizing system (Lorimer *et al.*, 1977). If the histone H1–polymer complex does function within the chromatin via a cross-linking mechanism, then the histone H1 within the complex must have the same or similar affinity for DNA within the chromatin as unmodified histone H1 in order to effect the cross-linking. Studies using DNA-cellulose chromatography have shown that unmodified histone H1 exhibits the same interaction with DNA as does the histone H1–polymer complex, which thus supports the cross-linking role for the histone H1–complex (Stone *et al.*, 1978). Also consistent with this hypothesis is the observation that there is an increased ADP-ribosylation in polynucleosomes more than three nucleosomes in length (Giri *et al.*, 1978*a*). Also, synthesis of the histone H1–poly(ADP-ribose) complex has recently been reported to occur in larger polynucleosomal structures (16 nucleosomes; quoted in Jump *et al.*, 1979).

The physiological significance of such histone modification *in vitro* must remain unclear at present, and although hypotheses and speculations have been made, one must wait for results from studies *in vivo* before an unambiguous assignment of the role that protein ADP-ribosylation plays in relation to chromatin structure and function can be made.

(3) *Conclusions*

Despite a great amount of research, ADP-ribosylation of nuclear proteins is, as yet, a poorly understood process. As section 2 showed, no clear-cut conclusion emerges as to the involvement of ADP-ribosylation in any one particular cellular function, with the possible exception of DNA repair. For elucidation of the role or roles fulfilled by a postsynthetic modification of proteins, the identification of the proteins modified is of paramount importance. The system is clearly heterogeneous; protein species that are known to be modified *in vivo* account for a small proportion of the total ADP-ribosylation observed. Although it is dangerous to draw on data obtained from studies *in vitro*, the results of Rickwood *et al.* (1977) suggest that over thirty protein species are modified. This may be either an overestimate (isolation of nuclei may expose new acceptor sites) or an underestimate (acceptors may be lost during isolation). The next step in the understanding of the role of ADP-ribosylation is the determination of the biological role played by the proteins that are ADP-ribosylated. The existence of two types of linkages between ADP-ribose and protein may indicate that at least two functions are fulfilled by ADP-ribosylation (as suggested by Bredehorst *et al.* (1979)). It is still unknown whether different enzymes are responsible for the formation of these different bonds or indeed if the initial ADP-ribose molecule is attached by the same enzyme that elongates the chain. Similarly, the relationship between mono(ADP-ribose) and poly(ADP-ribose) is unknown; is mono(ADP-ribosyl)ated protein a precursor or a degradation product of poly(ADP-ribosylated) protein, or are the two completely separate? If ADP-ribosylation serves only to modify an amino acid in an enzyme's active site or protein's binding site, why is the system capable of producing poly(ADP-ribose)?

Clearly, some approaches used to attempt the definition of the function of ADP-ribosylation are more likely to succeed than others. The large number of new analytical techniques to study ADP-ribosylation stems from the ambiguous results obtained with older methods. Thus the activities of poly(ADP-ribose) synthetase obtained from permeabilized cells are more meaningful than those obtained from isolated nuclei. Perhaps the most promising approach is to determine ADP-ribosylation of specific fractions of proteins throughout a cellular activity. The choice of a model system presents a problem, since changes in ADP-ribosylation usually occur during changes in cell status. Thus it is difficult to ascribe definitively an effect on a particular biological process. In veiw of this, it is noteworthy that an involvement in DNA repair seems to be a most likely candidate; it is also one of the easier systems to study *in vivo*. The use of inhibitor studies has been neglected. The main problem is the lack of specificity of compounds such as nicotinamide and thymidine. It is certain that the introduction of 3-amino- and 3-methoxy-benzamide, which are specific inhibitors of poly(ADP-ribose) synthetase (M. R. Purnell & W. J. D. Whish, unpublished work), should provide a useful probe for investigation.

Thus, despite many problems, a large number of sensitive analytical techniques are now available, and their use should rapidly increase our understanding of the cellular function of ADP-ribosylation.

Note Added in Proof. It has recently been shown by three groups that a purified poly(ADP-ribose) synthetase will both initiate and elongate poly(ADP-ribose) chains on exogenously added histones (see Purnell *et al.*, 1980).

We thank the Science Research Council (support to M. R. P., P. R. S. and W. J. D. W.), the Medical Research Council (support to W. J. D. W.) and Miles Laboratories (support to W. J. D. W. and M. P.). We also thank Pat Waller for typing the manuscript.

Adamietz, P. & Hilz, H. (1976) *Hoppe Seyler's Z. Physiol. Chem.* **357**, 527–534
Adamietz, P., Bredehorst, R. & Hilz, H. (1978*a*) *Eur. J. Biochem.* **91**, 317–326
Adamietz, P., Bredehorst, R. & Hilz, H. (1978*b*) *Biochem. Biophys. Res. Commun.* **81**, 1377–1383
Ballal, N. R. & Busch, H. (1973) *Cancer Res.* **33**, 2737–2743
Ballal, N. R., Goldknopf, I. L., Goldberg, P. A. & Busch, H. (1974) *Life Sci.* **14**, 1835–1845
Ballal, N. R., Kang, Y. J., Olson, M. O. J. & Busch, H. (1975) *J. Biol. Chem.* **250**, 5921–5925
Benjamin, R. C. & Gill, D. M. (1978) *J. Supramol. Struct. Suppl.* **2**, 74
Benjamin, R. C. & Gill, D. M. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 169
Berger, N. A. & Johnson, E. S. (1976) *Biochem. Biophys. Acta* **425**, 1–17
Berger, N. A., Weber, G. & Kaichi, A. S. (1978*a*) *Biochem. Biophys. Acta* **519**, 87–104
Berger, N. A., Weber, G., Kaichi, A. S. & Petzold, S. J. (1978*b*) *Biochem. Biophys. Acta* **519**, 105–117
Berger, N. A., Kaichi, A. S., Steward, P. G., Klevecz, R. R., Forrest, G. L. & Gross, S. D. (1978*c*) *Exp. Cell Res.* **117**, 127–135
Berger, N. A., Adams, J. W., Sikorski, G. W., Petzold, S. J. & Shearer, W. T. (1978*d*) *J. Clin. Invest.* **62**, 111–118
Berger, N. A., Sikorski, G. W., Petzold, S. J., Kurohara, K. K. (1979) *J. Clin. Invest.* **63**, 1164–1171
Bredehorst, R., Ferro, A. M. & Hilz, H. (1978*a*) *Eur. J. Biochem.* **82**, 105–113
Bredehorst, R., Ferro, A. M. & Hilz, H. (1978*b*) *Eur. J. Biochem.* **82**, 115–121
Bredehorst, R., Wielckens, K., Gartemann, A., Lengyel, H., Klapproth, K. & Hilz, H. (1978*c*) *Eur. J. Biochem.* **92**, 129–135
Burzio, L. O. & Koide, S. S. (1970) *Biochem. Biophys. Res. Commun.* **40**, 1013–1020
Burzio, L. O. & Koide, S. S. (1971) *Biochem. Biophys. Res. Commun.* **42**, 1185–1190
Burzio, L. O. & Koide, S. S. (1977) *Ann. N.Y. Acad. Sci.* **286**, 398–407
Burzio, L. O., Riquelme, P. T., Ohtsuka, E. & Koide, S. S. (1976) *Arch. Biochem. Biophys.* **173**, 306–319
Burzio, L. O., Riquelme, P. T. & Koide, S. S. (1979) *J. Biol. Chem.* **254**, 3029–3037
Butt, T. R., Brothers, J. F., Giri, G. P. & Smulson, M. E. (1978) *Nucleic Acids Res.* **5**, 2775–2788
Butt, T. R., Jump, D. B. & Smulson, M. E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1628–1632
Byrne, R. H., Stone, P. R. & Kidwell, W. R. (1978) *Exp. Cell. Res.* **115**, 277–283
Caplan, A. I. & Rosenberg, M. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1852–1857
Caplan, A. I., Ord, M. G. & Stocken, L. A. (1978) *Biochem. J.* **174**, 475–483

Chambon, P., Weill, J. D., Doly, J., Strosser, M. T. & Mandel, P. (1966) *Biochem. Biophys. Res. Commun.* **25**, 638–643
Clark, J. B., Ferris, G. M. & Pinder, S. (1971) *Biochem. Biophys. Acta* **238**, 82–85
Claycomb, W. C. (1976*a*) *FEBS Lett.* **61**, 231–233
Claycomb, W. C. (1976*b*) *Biochem. J.* **154**, 387–393
Cleaver, J. E. & Thomas, G. H. (1969) *Biochem. Biophys. Res. Commun.* **36**, 203–208
Colyer, R. A., Burdette, K. E. & Kidwell, W. R. (1973) *Biochem. Biophys. Res. Commun.* **53**, 960–965
Davies, M. I., Halldorssen, H., Shall, S. & Skidmore, C. J. (1976) *Biochem. Soc. Trans.* **4**, 635–637
Davies, M. I., Shall, S. & Skidmore, C. J. (1977) *Biochem. Soc. Trans.* **5**, 949–950
Dietrich, L. S., Jaus, H. & Siebert, G. (1973) *FEBS Lett.* **37**, 228–230
Ellison, R. P. (1978) *J. Cell Biol.* **79**, 131a
Farzaneh, F. & Pearson, C. K. (1977) *Biochem. Soc. Trans.* **5**, 733–734
Farzaneh, F. & Pearson, C. K. (1978) *Biochem. Biophys. Res. Commun.* **84**, 537–543
Ferro, A. M. & Oppenheimer, N. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 809–813
Ferro, A. M., Minaga, T., Piper, W. N. & Kun, E. (1978) *Biochem. Biophys. Acta* **519**, 291–305
Finch, J. T. & Klug, A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1897–1901
Fujimura, S., Hasegawa, S. & Sugimura, T. (1967) *Biochem. Biophys. Acta* **134**, 496–499
Furneaux, H. M. & Pearson, C. K. (1978) *Biochem. Soc. Trans.* **6**, 753–755
Futai, M., Mizuno, D. & Sugimura, T. (1967) *Biochem. Biophys. Res. Commun.* **28**, 395–399
Futai, M., Mizuno, D. & Sugimura, T. (1968) *J. Biol. Chem.* **243**, 6325–6329
Ghani, Q. P. & Hollenberg, M. (1978*a*) *Biochem. J.* **170**, 387–394
Ghani, Q. P. & Hollenberg, M. (1978*b*) *Biochem. Biophys. Res. Commun.* **81**, 886–891
Gill, D. M. & Meren, R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3050–3054
Giri, C. P., West, M. H. P. & Smulson, M. E. (1978*a*) *Biochemistry* **17**, 3495–3500
Giri, C. P., West, M. H. P., Ramirez, M. L. & Smulson, M. E. (1978*b*) *Biochemistry* **17**, 3501–3504
Goebel, M., Stone, P. R., Lengyel, H. & Hilz, H. (1977) *Hoppe Seyler's Z. Physiol. Chem.* **358**, 13–21
Goff, C. G. (1974) *J. Biol. Chem.* **249**, 6181–6190
Goldknopf, I. L. & Busch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 864–868
Gottesfeld, J. M., Garrard, W. T., Bagi, G., Wilson, R. F. & Bonner, J. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2193–2197
Gottesfeld, J. M., Murphy, R. F. & Bonner, J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4404–4408
Haines, M. E., Johnston, I. R., Mathias, A. P. & Ridge, D. (1969) *Biochem. J.* **115**, 881–887
Halldorsson, H., Gray, D. A. & Shall, S. (1978) *FEBS Lett.* **85**, 349–352
Hasegawa, S., Fujimura, S., Shimizu, Y. & Sugimura, T. (1967) *Biochem. Biophys. Acta* **149**, 369–376
Hashida, T., Ohgushi, H. & Yoshihara, K. (1979) *Biochem. Biophys. Res. Commun.* **88**, 305–311
Hayaishi, O. & Ueda, K. (1977) *Annu. Rev. Biochem.* **46**, 95–116
Hayaishi, O., Ueda, K., Kawaichi, M., Ogata, N., Ikai, K., Oka, J., Kim, H. R., Ito, S., Shizuta, Y. & Okayama, H. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 618
Hilz, H. & Kittler, M. (1971) *Hoppe Seyler's Z. Phys. Chem.* **352**, 1693–1704
Hilz, H. & Stone, P. R. (1976) *Rev. Physiol. Biochem. Pharmacol.* **76**, 1–58
Hogeboom, G. & Schneider, W. (1952) *J. Biol. Chem.* **197**, 611–620
Inagaki, F., Miyazawa, T., Miwa, M., Saito, H. & Sugimura, T. (1978) *Biochem. Biophys. Res. Commun.* **85**, 415–420
Ito, S., Shizuta, Y. & Hayaishi, O. (1979) *J. Biol. Chem.* **254**, 3647–3651
Jacobson, M. K. & Jacobson, E. L. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1347
Jacobson, E. L. & Narasimhan, G. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 619
Janakidevi, K. (1978) *Exp. Cell Res.* **112**, 345–351
Jump, D. B., Butt, T. R. & Smulson, M. E. (1979) *Biochemistry* **18**, 983–990
Kanai, Y., Miwa, M., Matsushima, T. & Sugimura, T. (1974) *Biochem. Biophys. Res. Commun.* **59**, 300–306
Kanai, Y., Kawaminami, Y., Miwa, M., Matsushima, T., Sugimura, T., Moroi, Y. & Yokohari, R. (1977) *Nature (London)* **265**, 175–177
Kanai, Y., Sugimura, T. & Matsushima, T. (1978) *Nature (London)* **274**, 809–812
Khan, M. G. & Shall, S. (1976) *Biochem. Soc. Trans.* **4**, 778–781
Kidwell, W. R. & Mage, M. (1976) *Biochemistry* **15**, 1213–1217
Kitamura, A., Tanigawa, Y., Yamamoto, T., Kawamura, M., Doi, S. & Shimoyama, Y. (1979) *Biochem. Biophys. Res. Commun.* **87**, 725–733
Kornberg, R. D. (1977) *Annu. Rev. Biochem.* **49**, 931–954
Kristensen, T. & Holtlund, J. (1976) *Eur. J. Biochem.* **70**, 441–446
Kristensen, T. & Holtlund, J. (1978) *Eur. J. Biochem.* **88**, 495–501
Kun, E., Chang, A. C. Y., Sharma, M. L., Ferro, A. M. & Nitecki, D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3131–3135
Lehmann, A. R. & Kirk-Bell, S. (1972) *Eur. J. Biochem.* **31**, 438–445
Levi, V., Jacobson, E. L. & Jacobson, M. K. (1978) *FEBS Lett.* **88**, 144–146
Lorimer, III, W. S., Stone, P. R. & Kidwell, W. R. (1977) *Exp. Cell Res.* **106**, 261–266
Mandel, P., Okazaki, H. & Niedergang, C. (1977) *FEBS Lett.* **84**, 331–336
Matinyan, K. S. & Umanskii, S. R. (1978) *Biokhimiya* **43**, 1196–1205
Matsubara, H., Hasegawa, S., Fujimura, S., Shima, T., Sugimura, T. & Futai, M. (1970) *J. Biol. Chem.* **245**, 3606–3611
McLachlan, J., Bateman, M. & Wolpert, L. (1976) *Nature (London)* **264**, 267–269
Minaga, T., Morton, L. J., Piper, W. N. & Kun, E. (1978) *Eur. J. Biochem.* **91**, 577–585
Miwa, M. & Sugimura, T. (1971) *J. Biol. Chem.* **246**, 6362–6364
Miwa, M., Tanaka, M., Matsushima, T. & Sugimura, T. (1974) *J. Biol. Chem.* **249**, 3475–3482
Miwa, M., Tanaka, M., Shinshi, H., Takeuchi, M., Matsushima, T., Sugimura, T. & Shall, S. (1975*a*) *J. Biochem. (Tokyo)* **77**, 3p
Miwa, M., Nakatsugawa, K., Hara, K., Matsushima, T. & Sugimura, T. (1975*b*) *Arch. Biochem. Biophys.* **167**, 54–60
Miwa, M., Oda, K., Segawa, K., Tanaka, M., Ine, S., Yamaguchi, N., Kuchino, T., Shiroki, K., Shimojo, H., Sakura, H., Matsushima, T. & Sugimura, T. (1977*a*) *Arch. Biochem. Biophys.* **181**, 313–321
Miwa, M., Saito, H., Sakura, H., Saikawa, N., Watanabe, F., Matsushima, T. & Sugimura, T. (1977*b*) *Nucleic Acids Res.* **4**, 3997–4005
Miwa, M., Saikawa, N., Yamaizumi, Z., Nishimura, S. & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 595–599
Miyakawa, N., Ueda, K. & Hayaishi, O. (1972) *Biochem. Biophys. Res. Commun.* **49**, 239–245
Morioka, K., Tanaka, K., Nokuo, T., Ishizawa, M. & Ono, T. (1979) *Gann* **70**, 37–46
Morton, R. K. (1958) *Nature (London)* **181**, 540–542
Muller, W. E. G. & Zahn, R. K. (1976*a*) *Experientia* **31**, 1014–1015
Muller, W. E. G. & Zahn, R. K. (1976*b*) *Mol. Cell Biochem.* **12**, 147–159
Muller, W. E. G., Totsuka, A., Nusser, I., Obermeier, J., Rhode, H. & Zahn, R. K. (1974) *Nucleic Acids Res.* **1**, 1317–1327
Muller, W. E. G., Falke, D., Zahn, R. K. & Arendes, J. (1979) *Intervirology* **11**, 182–187
Mullins, D. W., Giri, C. P. & Smulson, M. E. (1977) *Biochemistry* **16**, 506–513
Niedergang, C., Okazaki, H. & Mandel, P. (1978) *Anal. Biochem.* **88**, 20–28
Nishizuka, Y., Ueda, K., Nakazawa, K. & Hayaishi, O. (1967) *J. Biol. Chem.* **242**, 3164–3171
Nishizuka, Y., Ueda, K., Honjo, T. & Hayaishi, O. (1968) *J. Biol. Chem.* **243**, 3765–3767
Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M. & Hayaishi, O. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 781–786
Nolan, N. (1978) Ph.D. Thesis, the Catholic University of America, Washington D.C.
Okayama, H. & Hayaishi, O. (1978) *Biochem. Biophys. Res. Commun.* **84**, 755–762
Okayama, H., Edson, D. M., Fukushima, M., Ueda, K. & Hayaishi, O. (1977) *J. Biol. Chem.* **252**, 7000–7005
Okayama, H., Ueda, K., & Hayaishi, O. (1978*a*) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1111–1115

Okayama, H., Honda, M. & Hayaishi, O. (1978*b*) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2254–2257
Okazaki, H., Niedergang, C. & Mandel, P. (1976) *FEBS Lett.* **62**, 255–258
Okolie, E. E. & Shall, S. (1979) *Clin. Exp. Immunol.* **36**, 151–164
Ord, M. G. & Stocken, L. A. (1977) *Biochem. J.* **161**, 583–592
Perella, F. W. & Lea, M. A. (1978) *Biochem. Biophys. Res. Commun.* **82**, 575–581
Perella, F. W. & Lea, M. A. (1979) *Cancer Res.* **39**, 1382–1389
Pesce, A., Casoli, C. & Schito, G. C. (1976) *Nature (London)* **262**, 412–414
Poirier, G. G., Savarel, P., Rojotte, D., Morisset, J. & Lord, A. (1978) *Can. J. Biochem.* **56**, 784–790
Preiss, J., Schlaeger, R. & Hilz, H. (1971) *FEBS Lett.* **19**, 244–246
Purnell, M. R., Stone, P. R., Surowy, C. J. & Whish, W. J. D. (1980) *Proceedings of Conference on Novel ADP-ribosylations of Regulatory Enzymes and Proteins*, National Institutes of Health, Washington and Elsevier, New York, in the press
Rastl, E. & Swetly, P. (1978) *J. Biol. Chem.* **253**, 4333–4340
Rechsteiner, M. & Catanzarite, V. (1974) *J. Cell. Physiol.* **84**, 409–422
Rechsteiner, M., Hillyard, D. & Olivera, B. M. (1976) *Nature (London)* **259**, 695–696
Reeder, R. H., Ueda, K., Honjo, T., Nishizuka, Y. & Hayaishi, O. (1967) *J. Biol. Chem.* **242**, 3172–3179
Renz, M., Nehls, P. & Holzer, J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1879–1883
Rickwood, D., MacGillivray, A. J. & Whish, W. J. D. (1977) *Eur. J. Biochem.* **79**, 589–598
Riquelme, P., Burzio, L. O. & Koide, S. S. (1979) *J. Biol. Chem.* **254**, 3018–3028
Roberts, J. H., Stark, P. & Smulson, M. E. (1973) *Biochem. Biophys. Res. Commun.* **52**, 43–50
Rohrer, H., Zillig, W. & Mailhammer, R. (1975) *Eur. J. Biochem.* **60**, 227–238
Roitt, I. M. (1956) *Biochem. J.* **63**, 300–307
Sakura, H., Miwa, M., Tanaka, M., Kanai, Y., Shimada, T., Matsushima, T. & Sugimura, T. (1977) *Nucleic Acids Res.* **4**, 2903–2915
Sakura, H., Miwa, M., Kanai, Y., Matsushima, T. & Sugimura, T. (1978) *Nucleic Acids Res.* **5**, 4025–4083
Schacter, L. P. & Burke, P. J. (1978) *Biochem. Biophys. Res. Commun.* **80**, 504–510
Shall, S., Goodwin, P., Halldorsson, H., Khan, G., Skidmore, C. & Tsopanakis, C. (1977) *Biochem. Soc. Symp.* **42**, 103–116
Shinsi, H., Miwa, M., Kato, K., Naguchi, M., Matsushima, T. & Sugimura, T. (1976) *Biochemistry* **15**, 2185–2190
Sims, J. L., Juarez, H. & Jacobson, M. K. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 493
Skorko, R., Zillig, W., Rohrer, H., Fujiki, H. & Mailhammer, R. (1977) *Eur. J. Biochem.* **79**, 55–66
Smith, J. A. & Stocken, L. A. (1973) *Biochem. Biophys. Res. Commun.* **54**, 297–300
Smith, J. A. & Stocken, L. A. (1975) *Biochem. J.* **147**, 523–529
Smulson, M. E., Stark, P., Gazzoli, M. & Roberts, J. (1975) *Exp. Cell Res.* **90**, 175–182
Smulson, M. E., Schein, P., Mullins, D. W. & Sudhakar, S. (1977) *Cancer Res.* **37**, 3006–3012
Stone, P. R. & Hilz, H. (1975) *FEBS Lett.* **57**, 209–212
Stone, P. R., Whish, W. J. D. & Shall, S. (1973) *FEBS lett.* **36**, 334–337
Stone, P. R., Bredehorst, R., Kittler, M., Lengyel, H. & Hilz, H. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 51–56
Stone, P. R., Lorimer III, W. S. & Kidwell, W. R. (1977) *Eur. J. Biochem.* **81**, 9–18
Stone, P. R., Lorimer III, W. S., Ranchalis, J., Danley, M. & Kidwell, W. R. (1978) *Nucleic Acids Res.* **5**, 173–184
Stratling, W. H., Muller, U. & Zentgraf, H. (1978) *Exp. Cell Res.* **117**, 301–311
Sudhakar, S., Tew, K. D. & Smulson, M. E. (1979*a*) *Cancer Res.* **39**, 1405–1410
Sudhakar, S., Tew, K. D., Schein, P. S., Woolley, P. V. Smulson, M. E. (1979*b*) **39**, 1411–1415
Sugimura, T. (1973) *Prog. Nucleic Acid Res. Mol. Biol.* **13**, 127–151
Sugimura, T., Yoshimura, N., Miwa, M., Nagai, H. & Nagao, M. (1971) *Arch. Biochem. Biophys.* **147**, 660–665
Suhadolnik, R. J., Baur, R., Lichtenwalner, D. M., Uematsu, T., Roberts, J. H., Sudhakar, S. K. & Smulson, M. E. (1977) *J. Biol. Chem.* **252**, 4134–4144
Surowy, C. J. & Whish, W. J. D. (1980) *Biochem. Soc. Trans.* **8**, 174–175
Suzuki, Y. & Murachi, T. (1978) *J. Biochem. (Tokyo)* **84**, 977–984
Tanaka, M., Miwa, M., Matsushima, T., Sugimura, T. & Shall, S. (1976) *Arch. Biochem. Biophys.* **172**, 224–229
Tanaka, M., Miwa, M., Hayashi, K., Kubota, K., Matsushima, T. & Sugimura, T. (1977) *Biochemistry* **16**, 1485–1489
Tanaka, M., Hayashi, K., Sakura, H., Miwa, M., Matsushima, T. & Sugimura, T. (1978) *Nucleic Acid Res.* **5**, 3183–3194
Tanigawa, Y., Kawamura, M. & Shimoyama, M. (1977) *Biochem. Biophys. Res. Commun.* **76**, 406–412
Tanigawa, Y., Kawamura, M., Kitamura, A. & Shimoyama, M. (1978*a*) *Biochem. Biophys. Res. Commun.* **81**, 1278–1285
Tanigawa, Y., Kitamura, A. & Shimoyama, M. (1978*b*) *Eur. J. Biochem.* **92**, 261–269
Tanuma, S., Enomoto, T. & Yamada, M. (1977) *Biochem. Biophys. Res. Commun.* **74**, 599–605
Tanuma, S., Enomoto, T. & Yamada, M. (1978) *Exp. Cell Res.* **117**, 421–430
Thoma, F. & Koller, Th. (1977) *Cell* **12**, 101–107
Tsopanakis, C., MacLaren, E. A. & Shall, S. (1976) *Biochem. Trans.* **4**, 774–777
Tsopanakis, C., MacLaren, E. A., Tsopanakis, A. & Shall, S. (1977) *Biochem. Soc. Trans.* **5**, 932–936
Tsopanakis, C., MacLaren, E. A., Tsopanakis, A. & Shall, S. (1978*a*) *Eur. J. Biochem.* **90**, 337–345
Tsopanakis, C., Leer, J. C., Nielsen, O. F., Gocke, E., Shall, S. & Westergaard, O. (1978*b*) *FEBS Lett.* **93**, 297–300
Ueda, K., Oka, J., Narumiya, S., Miyakawa, N. & Hayaishi, O. (1972) *Biochem. Biophys. Res. Commun.* **46**, 516–523
Ueda, K., Omachi, A., Kawaichi, M. & Hayaishi, O. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 205–209
Ueda, K., Kawaichi, M., Okayama, H. & Hayaishi, O. (1979) *J. Biol. Chem.* **254**, 679–687
Whish, W. J. D., Davies, M. I. & Shall, S. (1975) *Biochem. Biophys. Res. Commun.* **65**, 722–730
Whitby, A. J. & Whish, W. J. D. (1977) *Biochem. Soc. Trans.* **5**, 948–949
Whitby, A. J. & Whish, W. J. D. (1978) *Biochem. Soc. Trans.* **6**, 619–620
Whitby, A. J., Stone, P. R. & Whish, W. J. D. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1295–1304
Wielckens, K., Sachsenmaier, W. & Hilz, H. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 39–43
Wong, N. C., Poirier, G. G. & Dixon, G. H. (1977) *Eur. J. Biochem.* **77**, 11–21
Worcel, A. & Benyajati, C. (1977) *Cell* **12**, 83–100
Yamada, M., Shimada, T., Nakayasu, M., Okada, H. & Sugimura, T. (1978) *Biochem. Biophys. Res. Commun.* **83**, 1325–1332
Yoshihara, K., Tanigawa, Y., Burzio, L. O. & Koide, S. S. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 289–293
Yoshihara, K., Hashida, T., Yoshihara, H., Tanaka, Y. & Ohgushi, H. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1281–1288
Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H. & Kamiya, T. (1978) *J. Biol. Chem.* **253**, 6459–6466
Young, R. J. & Sweeney, K. (1978) *Biochemistry* **17**, 1901–1907
Young, R. J. & Sweeney, K. (1979) *J. Embryol. Exp. Morph.* **49**, 139–152
Yuki, H., Sempuku, C., Park, M. & Takiura, K. (1972) *Anal. Biochem.* **46**, 123–128
Yukioka, M., Oka, Y., Hasuma, T. & Inoue, A. (1978) *FEBS Lett.* **86**, 85–88

Smulson, Sugimura, eds. Novel ADP-Ribosylations
of Regulatory Enzymes and Proteins

ADP-RIBOSYLATION OF NUCLEAR PROTEINS IN VIVO

MICHAEL PURNELL, PETER STONE, CAROL SUROWY AND WILLIAM WHISH
Biochemistry Group, University of Bath, Bath, U.K.

INTRODUCTION

A variety of functions have been ascribed to poly(ADP-ribose)[1,2](for a more recent review from our laboratory see [3]), although the biological role of the polymer is unknown. Before any attempt to determine the physiological significance of poly(ADP-ribose), or more importantly the role of the modified proteins, can be made, a non-selective and quantitative isolation of ADP-ribosylated protein must be developed. It is such methodology which will be applicable to a wide variety of systems, which we report here.

MATERIALS AND METHODS

Mouse LS1210 cells were grown at 37°C under 5% ($^{v}/_{v}$) CO_2 in air in suspension culture in Minimal Essential Medium supplemented with 10% ($^{v}/_{v}$) horse serum, 2mM glutamine and 100I.U./1ml each of penicillin and streptomycin. Cells were labelled with [2-^{3}H]adenosine (24Ci/mmole) at a concentration of 40μCi/ml for 16 hours prior to processing for CsCl-urea equilibrium density gradient centrifugation. Cells were harvested and washed once with phosphate buffered saline, quickly frozen and resuspended in 70% ethanol-50mM acetate buffer pH 5. The samples were centrifuged at 1,000xg for 20 minutes and the pellet washed once with 70% ethanol-acetate and twice with 60% ethanol-50mM acetate pH 5. The pellet was finally resuspended in 5 ml of 5M urea, 50% ($^{w}/_{w}$) CsCl, 0.1mM PMSF, 0.1M sodium acetate pH 5 and centrifuged at 45,000 r.p.m. in a Beckman SW 50.1 rotor for 72 hours at 4°C. The gradients were fractionated and the acid insoluble [^{3}H]-adenosine determined using the filter disc assay as previously described[4].

The top fractions containing ADP-ribosylated were pooled, T.C.A. added to a final concentration of 25% ($^{w}/_{v}$) and the samples left on ice for 4 hours. The acid precipitable material was removed by centrifugation at 38,000xg for 15 minutes and washed three times with 25% ($^{w}/_{v}$) T.C.A. and twice with ethanol-50mM acetate pH 5. The average chain lengths were determined by the method of Nishizuka et al[5] following digestion with snake venom phosphodiesterase[4] and PEI-cellulose thin layer chromatography as described previously[6] using the solvent system of Randerath and Randerath[7]. For further characterisation, the material was treated with 0.1M NaOH at 37°C overnight and neutralised with

with acetic acid. The radioactive products were analysed using thin layer chromatography. 5'-AMP and 3'-AMP were resolved using the borate system of Schwartz and Drach[8]. Following extensive digestion of the material with snake venom phosphodiesterase and alkaline phosphatase, adenosine and ribosyl adenosine were separated by the method of Miwa et al[9]. Determination of the presence of deoxyadenosine (derived from DNA) was performed using t.l.c.[10]

RESULTS

As can be seen from Fig. 1A the methodology described separates DNA and RNA from protein. Analysis of the adenosine labelled cells shows the distribution of radioactivity to be between DNA/RNA and protein (Fig. 1B).

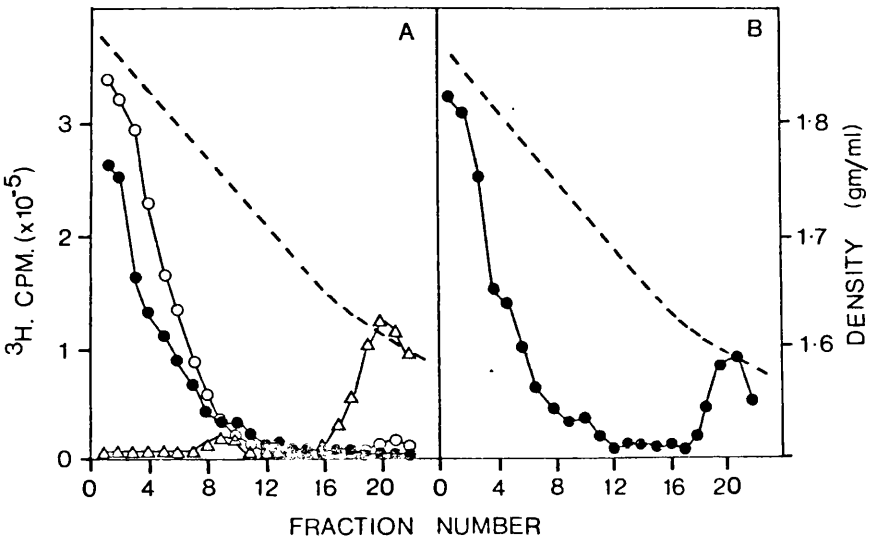


Fig. 1. Separation of DNA, RNA and protein. Cells were grown for 18 hours in the presence of [Methyl-^{3}H]thymidine (●), [5-^{3}H]uridine (○), L-[4,5-^{3}H]leucine (△) (1A); or [2-^{3}H]adenosine (●) (1B) and the labelled material analysed by equilibrium density gradient centrifugation as described in Materials and Methods. All isotopes were added at 40μCi/ml culture.

Initially, the protein associated label was analysed by a variety of enzymic digestions as well as chemical analysis. The results (Table 1) show that the radioactivity is predominantly mono- or poly- ADP-ribose as evidenced by its susceptibility to snake venom phosphodiesterase and its resistance to DNase I, ribonuclease and spleen phosphodiesterase. As would be expected for ADP-

ribosylated protein the labelled material also shows susceptibility to NH_2OH, pronase and NaOH. T.l.c. analysis[7] revealed that approximately 20% of the NH_2OH released adenosine label migrated as ADP-ribose (data not shown). Conversion of the material to nucleosides by digestion with DNase I, spleen and snake venom phosphodiesterase and alkaline phosphatase demonstrated the complete absence of deoxyadenosine and thus DNA in the preparation.

TABLE 1

ENZYMIC AND CHEMICAL ANALYSIS OF [^{3}H]ADENOSINE LABELLED MATERIAL

TREATMENT	% RADIOACTIVITY DIGESTED*
CONTROL	3
SNAKE VENOM PHOSPHODIESTERASE	92
SNAKE VENOM P.D.E. + ALKALINE PHOSPHATASE	93
SPLEEN PHOSPHODIESTERASE	17
RIBONUCLEASE A	14
DEOXYRIBONUCLEASE I	2
PRONASE (1mg/ml, 2% SDS)	68
SODIUM HYDROXIDE (0.1N)	87
HYDROXYLAMINE (0.8M pH 7.5)	21

The T.C.A. precipitated [^{3}H]adenosine material was treated as indicated in the table. All incubations were performed at 37^{o}C and the reactions followed using the filter disc assay[4] until completion. Enzymes were added at one I.U./ml digest except for pronase (1mg/ml) and RNase and DNase which were added 100kunitz units/ml.

The presence of approximately 15% RNA is suggested by the ribonuclease A and spleen phosphodiesterase digestions (Table 1). To verify this, the acid insoluble material was treated with NaOH, which has been shown to completely remove poly(ADP-ribose) and mono-ADP-ribose from protein[11] and the products analysed (Table 2).

Thin layer analysis demonstrated that 14-18% of the radioactivity migrated as 3'-AMP (or 2'-AMP), the breakdown product of RNA. Over 50% of the material migrated as 5'-AMP, the specific product of mono-ADP-ribose hydrolysis[12]. Enzymic digestion of the base released AMP with either 5'- or 3'-nucleotidase confirmed the above distribution. The radioactivity remaining on the origin of the t.l.c. is poly(ADP-ribose) because prior digestion with snake venom phosphodiesterase results in the generation of PR-AMP and AMP with a concomitant loss of the origin material.

TABLE 2

ANALYSIS OF NaOH HYDROLYSATE OF [^{3}H]ADENOSINE LABELLED MATERIAL BY THIN LAYER CHROMATOGRAPHY

METHOD OF ANALYSIS (T.L.C. REFERENCE)	% RADIOACTIVITY ON T.L.C. ORIGIN	5'-AMP	3'-AMP	ADENOSINE
8	24	52	18	0
1M ACETIC ACID (4cm) + 0.3M LiCl (8cm)[7]	21.5	57	14	0
3'-NUCLEOTIDASE + 6,7	22.5	54.5	0	20
5'-NUCLEOTIDASE + 6,7	21	0	17	59

The average chain length of the protein associated $(ADP\text{-}ribose)_n$ was determined and found to be 1.2 (Table 3). Since 15% of the AMP is 3'-AMP and derived from RNA (Table 2) the appropriately corrected value for 5'-AMP gave a value of 1.5. The chain length of the oligomeric ADP-ribose was calculated (ie, the origin material) by a further correction for the mono-ADP-ribose derived 5'-AMP to give a value of 3.3 (Table 3). This was verified experimentally by determining the average chain length of the base released origin material after removal of the AMP by ion-exchange chromatography. This characterisation has thus demonstrated the presence in vivo of both monomeric and oligomeric protein-bound ADP-ribose. Furthermore, the methodology has been shown to be applicable to analysis of ADP-ribosylated proteins in rat pancreas[13] and germinating wheat embryo[14].

TABLE 3

AVERAGE CHAIN LENGTH ANALYSIS OF [^{3}H]ADENOSINE LABELLED MATERIAL

TREATMENT	T.L.C. ANALYSIS (REFERENCE)	AVERAGE CHAIN LENGTH FOUND	CORRECTED
SNAKE VENOM PHOSPHODIESTERASE	6,7	1.2	1.5* 3.3**
SNAKE VENOM PHOSPHODIESTERASE + ALKALINE PHOSPHATASE	9	1.21	1.5*

* Corrected for 3'-AMP ** Corrected for 3'-AMP and 5'-AMP

Compounds which are known to inhibit poly(ADP-ribose) synthesis *in vitro* have been used extensively to study the function of poly(ADP-ribose) *in vivo* although no direct evidence has been presented to show that poly(ADP-ribose) synthesis is inhibited *in vivo*. Thus it was of considerable interest to apply the above methodology to a study of inhibition of poly(ADP-ribose) synthetase *in vivo*. The effect of thymidine[15] and 3-aminobenzamide[16] on ADP-ribosylation in mouse L1210 was therefore examined. The results are presented in Table 4 and it can be seen that both compounds result in the complete inhibition of poly(ADP-ribose) synthesis. Confirmation of this finding was achieved by the analysis of the base hydrolysed radioactive material from cells grown in the presence of inhibitor which showed the absence of radioactivity on the origin (Table 5).

TABLE 4

EFFECT OF INHIBITORS ON POLY(ADP-RIBOSE) SYNTHESIS IN VIVO

TREATMENT	RADIOACTIVITY ON T.L.C. PR-AMP	AMP
CONTROL	300	1500
THYMIDINE	25	1350
3-AMINOBENZAMIDE	20	1395

Cells were grown in the presence of [^{3}H]adenosine ± inhibitors, processed as described in methods and the snake venom phosphodiesterase digest analysed[6,7].

TABLE 5

EFFECT OF INHIBITORS ON [^{3}H]ADENOSINE LABELLED MATERIAL

TREATMENT	% RADIOACTIVITY ON T.L.C. ORIGIN	5'-AMP	3'-AMP
CONTROL	25	55	20
THYMIDINE (5mM)	0	75	22
3-AMINOBENZAMIDE (2mM)	0	75	23

Cells were grown in the presence of [^{3}H]-adenosine ± inhibitors, processed as described in methods and the NaOH released material analysed[8].

These results demonstrate, for the first time, that known inhibitors of poly(ADP-ribose) synthetase *in vitro* do indeed completely inhibit polymer formation *in vivo*. Previous evidence for the effects of inhibitors *in vivo* is by inference; the decrease in cellular NAD levels observed upon treatment of cells with DNA damaging agents is abolished by co-administration of known *in vitro* inhibitors of poly(ADP-ribose) synthetase[17]. Furthermore, a recent study suggests that thymidine inhibits ADP-ribosylation *in vivo* as evidenced by the stimulation of poly(ADP-ribose) synthetase activity in nuclei isolated from cultured cells synchronised by treatment with thymidine as opposed to hydroxyurea[18]. The only previous direct attempt to demonstrate inhibition of polymer synthesis *in vivo* following administration of an inhibitor (nicotinamide) showed an increased level of endogenous poly(ADP-ribose) in rat heart and liver[19]. Such an observation highlights the need to use specific inhibitors for *in vivo* studies since administration of nicotinamide to rats is known to result in elevated levels of NAD in the liver[20].

Although it can be seen that addition of inhibitors to cultures of mouse L1210 cells abolishes the synthesis of poly(ADP-ribose) (Table 4,5) it is of interest that the synthesis of mono ADP-ribose appears to be unaffected (Table 5). These determinations are not quantitative and absolute values for the levels of mono ADP-ribose in control and inhibitor-treated cells are needed before a definitive interpretation can be made. It is tempting, however, to speculate on the origin of this mono ADP-ribose synthesised in the presence of inhibitors. One possibility is that there are at least two synthetic enzymes involved in ADP-ribosylation of proteins, one or more responsible for initiation and the other(s) which extend the monomer to form polymer, and that initiation is not affected by the inhibitors. This seems likely in view of the observation that histones, which are known acceptors for ADP-ribosylation *in vivo*[21], are not ADP-ribosylated in a reconstituted system containing highly purified poly(ADP-ribose) synthetase[22]. The purified enzyme preparation, however, will ADP-ribosylate a histone H1-mono ADP-ribose adduct[23].

In this communication we have demonstrated the presence *in vivo* of mono- and poly ADP-ribosylated proteins and, furthermore, have fully characterised both the monomer and polymer. The separation of such ADP-ribosylated proteins from DNA and RNA will enable the subsequent identification and characterisation of those proteins which are modified *in vivo*.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of the Medical Research Council, Science Research Council and Miles Laboratories.

REFERENCES

1. Hayaishi, O. and Ueda, K. (1977) Ann. Rev. Biochem. 46, 95-116.
2. Hilz, H. and Stone, P.R. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1-58.
3. Purnell, M.R., Stone, P.R. and Whish, W.J.D. (1980) Biochem. Soc. Trans. In Press.
4. Whitby, A.J., Stone, P.R. and Whish, W.J.D. (1979) Biochem. Biophys. Res. Commun. In Press.
5. Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda M. and Hayaishi, O. (1969) Cold Spring Harbor Sym. Quant. Biol. 34, 781-786.
6. Stone, P.R., Whish, W.J.D. and Shall, S. (1973) FEBS Lett. 36, 334-337.
7. Randerath, K. and Randerath, E. (1967) Methods in Enzymol. (Colowick, S.P. and Kaplan, N.O. eds) Vol. 12A, pp.323-347, Academic Press, New York.
8. Schwartz, P.M. and Drach, J.C. (1975) J. Chromatog. 106, 200-203.
9. Miwa, M., Saikawa, N., Yamaizumi, Z., Nishimura, S. and Sugimura, T. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 595-599.
10. Randerath, E. and Randerath, K. (1965) Anal. Biochem. 12, 83-93.
11. Adamietz, P. and Hilz, H. (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 527-534.
12. Goebel, M., Stone, P.R., Lengyel, H. and Hilz, H. (1977) Hoppe Seyler's Z. Physiol. Chem. 358, 13-21.
13. Whish, W.J.D. and Poirier, G.G. (1980) 586th Biochemical Society Meeting, Bristol, Biochem. Soc. Trans. In Press.
14. Abed, N. and Whish, W.J.D. - unpublished data.
15. Preiss, J., Schlaeger, R. and Hilz, H. (1971) FEBS Lett. 19, 244-246.
16. Purnell, M.R. and Whish, W.J.D. (1979) Biochem. J. submitted.
17. Davies, M.I., Halldorsson, H., Shall, S. and Skidmore, C.J. (1976) Biochem. Soc. Trans. 4, 635-637.
18. Tanuma, S., Enomoto, T. and Yamada, M. (1979) J. Biol. Chem. 254, 4960-4962.
19. Ferro, A.M., Minaga, T., Piper, W.N. and Kun, E. (1978) Biochim. Biophys. Acta. 519, 291-305.
20. Kaplan, N.O., Goldin, A., Humphreys, S.R. and Stolzenbach, F.E. (1957) J. Biol. Chem. 226, 365-371.
21. Ueda, K., Omachi, A., Kawaichi, M. and Hayaishi, O. (1975) Proc. Natl. Acad. Sci. (U.S.A.) 72, 205-209.
22. Oyakama, H., Edson, C.M., Tukushima, M., Ueda, K. and Hayaishi, O. (1977) J. Biol. Chem. 252, 7000-7005.
23. Ueda, K., Kawaichi, M., Okayama, H. and Hayaishi, O. (1979) J. Biol. Chem. 254, 679-687.

(See DISCUSSION on following page)

DISCUSSION

DR. BUTT: Since you start your experiment on Thursday, and start the gradient on Friday, and analyze the gradient on Monday, I really want to know, since you rely on the adenosine label at the top of the gradient, how much loss occurs since the zero time. Have you worked that out?

DR. STONE: Well, we get a quantitative recovery of the label. The proteins are all there. If there were degradation during centrifugation you would certainly expect to see smearing. I think the resolution is fairly nice. We go almost right down to base line.

DR. OGATA: I wonder if you have an accurate way to measure any polymer released from the protein as it goes down the gradient.

DR. STONE: Free polymer goes to the bottom.

DR. OGATA: What was the pH and temperature of the gradient?

DR. STONE: The pH was about 4 to 5, and the temperature was 4°C.

DR. MANDEL: From what we see, the gradient works very well. We are going to try the same thing, but in order to determine the amount of monomer and dimer we are going to measure fluorescence, so we have a specific activity.

DR. STONE: Our results are clearly not quantitative, but the interesting thing is that an inhibitor wipes out the polymer, and the cells still grow. So I think one should bear in mind that maybe the polymer *per se* is not so important as the monomer, and that the function may be related to a monitoring type role instead of a specific point in time where something has to happen. I think one should think of it as a sort of cleaning up process, where the modifications are sort of monitoring. I think in that respect, DNA is a very good candidate.

DR. HILZ: I'm not completely agreed with your conclusion that you can wipe out polymers, that you have no polymer lower down in the gradient. This fraction you would not see; you would not analyze. You have restricted your analysis to fractions of ADP-ribosylated proteins with a certain density, which means proteins modified to only a certain extent.

DR. STONE: But if you look at figure 1A, that's the profile with leucine, the protein is all at the top. The fact that there is no adenosine there doesn't matter. There is still endogenous material there, and all the protein is at the top.

DR. HILZ: Less than 1% would be lost.

DR. JACOBSON: I'm not certain that protein with thirty to forty residues of ADP-ribose would be at the top.

DR. STONE: The protein is at the top, but I can't talk about 1%.

DR. JACOBSON: But that is what I submit would be the expected amount of protein having poly(ADP-ribose) in the cells.

DR. BURZIO: You precipitate the top fractions with TCA and then wash with alcohol. Do you know how much you lose with the alcohol wash?

DR. STONE: Yes. There is a maximum loss of 1% to 2% of the acid precipitable material. So by the nature of the material at the top of the gradient, it must still be attached to protein to be acid precipitable.

DR. OLIVERA: If I understand the interpretation of what you are saying, the polymerization can be inhibited, but the cells still grow.

DR. STONE: Yes.

DR. OLIVERA: I am amazed that so much of the acid precipitable material is at the top of the gradient. Can you explain?

DR. STONE: We kill the cells as quickly as we can. We freeze them in liquid nitrogen. I think that is why we obtain so much label still attached to protein.

DR. OGATA: Is it possible that you are looking at the turnover of terminal adenosine on an already long chain?

DR. STONE: No. We have labeled overnight with adenosine and chased with cold adenosine. The half life seems to be one or two hours, so with 16 hours of labeling there should be little unlabeled material.

BIOCHEM SOC TRANS. 9 (in the press)

Application of aminoethyl cellulose chromatographic separation of monomeric and polymeric ADP-ribose

Michael R. Purnell, Peter R. Stone, Carol S. Surowy and William J.D. Whish

Biochemistry Group, School of Biological Sciences, University of Bath, Claverton Down, Bath, Avon BA2 7AY.

The biological role of nuclear protein ADP-ribosylation (for a recent review see Purnell et al., 1980a), although implicated in a wide variety of cellular processes, is not understood. It is clear, however, that in vivo and in vitro both mono and poly ADP-ribosylation of nuclear proteins, especially the histones, occurs (Purnell et al., 1980a,b) although the relationship between mono and poly ADP-ribose has not been established. Thus a simple and rapid analytical system is needed which will allow separation of mono and poly ADP-ribose in order to make conclusions regarding the extent of mono and poly ADP-ribosylation of specific nuclear proteins. It is such methodology which we wish to report in this present communication.

One of the intriguing properties of protein-ADP-ribose conjugates are their differing sensitivities towards neutral hydroxylamine. Thus, it has been shown that both mono and poly ADP-ribose are attached to proteins by at least two linkages. One of these is quantitatively cleaved by NaOH and neutral hydroxylamine, the other being NaOH sensitive and hydroxylamine resistant (Adamietz and Hilz, 1976). Of particular interest is histone H1 which is the most extensively studied acceptor to date (Purnell et al., 1980a) although no complete analysis of the extent of mono and poly ADP-ribosylation and their associated linkages has been made. Here we report such a detailed analysis.

A 0.5M NaCl extract of porcine thymus nuclei was incubated with [adenine-^{3}H]NAD$^+$ as previously described (Purnell and Whish, 1980) and histone H1 isolated by the perchloric acid extraction procedure of Johns (1964). The H1 was further purified to homogeneity (as judged by a single band on both acid-urea (Panyim and Chalkley, 1969) and SDS polyacrylamide gels (Weber and Osborn, 1969)) by

preferential precipitation with HCl and acetone (Goodwin and Johns, 1977). Greater than 95% of the radioactivity incorporated into the purified H1 preparation migrated as a single peak in association with the H1 on acid-urea polyacrylamide gels. Treatment of such H1 preparations with 1M ethylamine for 30 minutes at 37°C quantitatively cleaves the ADP-ribose-H1 linkage(s) as evidenced by a complete lack of any radioactivity electrophoresing with H1 on acid-urea polyacrylamide gels following such treatment. Analysis of the released radioactivity was performed using aminoethyl cellulose column chromatography. The ethylamine treated H1 was lyophilised to remove the ethylamine and then dissolved in 1ml water and applied to a column of aminoethyl cellulose (10 x 15mm). The column was eluted with 10ml 6N acetic acid then with 5ml water and finally with 10ml 1M ethylamine. The acetic acid and ethylamine fractions were then lyophilised and analysed by PEI-cellulose thin layer chromatography (Stone et al., 1973). The results show that mono ADP-ribose and its base derived products 5'-AMP and "X", an ADP-like compound (Goebel et al., 1977) elute exclusively with 6N acetic acid. No radioactivity is seen on the origin or between the origin and ADP-ribose on the thin layer chromatogram indicating an absence of poly ADP-ribose in the acetic acid fraction. The poly(ADP-ribose) elutes with 1M ethylamine from the aminoethyl cellulose and chromatographs on the origin and between the origin and ADP-ribose on PEI-cellulose. Thus, aminoethyl cellulose chromatography facilitates a complete separation of mono and poly ADP-ribose.

A labelled H1 preparation was incubated with 0.4M hydroxylamine, pH 7.0, for 30 minutes at 37°C and the released mono and poly ADP-ribose separated from both unmodified H1 and hydroxylamine resistant H1 conjugates by chromatography on Bio Rex 70 (Stone et al., 1977). Analysis of such H1 preparations by acid-urea polyacrylamide gel electrophoresis reveals that approximately 27% of the total H1-associated radioactivity is hydroxylamine resistant. This resistant material has also been analysed for mono and poly ADP-ribose by ethylamine treatment and aminoethyl cellulose chromatography. The results show that 40%

of the total H1-associated radioactivity is mono ADP-ribose and that approximately 13% of this monomer is hydroxylamine resistant. Of the 60% poly ADP-ribose attached to H1 37% is hydroxylamine resistant. These results show that H1 conjugates, synthesised in vitro in a porcine thymus nuclear salt extract, contain both hydroxylamine resistant and sensitive linkages to mono and poly ADP-ribose.

This methodology has also been applied to a study of proteins modified in vivo. Mouse L1210 cells were grown as previously described in [^{3}H]adenosine and the ADP-ribosylated proteins isolated by CsCl-urea density gradient centrifugation (Purnell et al., 1980b). The isolated proteins were treated with 1M ethylamine at 37^{o}C overnight and analysed by aminoethyl cellulose chromatography as described above for H1. The results show that of the total in vivo ADP-ribosylation approximately 73% is mono ADP-ribose and 27% is poly ADP-ribose. Furthermore, treatment of the ethylamine eluting poly ADP-ribose with snake venom phosphodiesterase and estimation of the average chain length (Nishizuka et al., 1969) reveals a value of 2.92. Obviously it would be of interest to know the extent of hydroxylamine resistant mono and poly ADP-ribosylation in vivo as well as the average chain length of such resistant poly ADP-ribose and we are currently engaged in such studies.

We thank the Science Research Council (support to MRP, PRS and WJDW), the Medical Research Council (support to CSS and WJDW) and Miles Laboratories (support to MRP and WJDW).

ANALYTICAL BIOCHEMISTRY 109, 000–000 (1980)

A Method for Analyzing the ADP-Ribosylation of Nuclear Proteins on Polyacrylamide Gels

PETER R. STONE, MICHAEL R. PURNELL, AND WILLIAM J. D. WHISH

Department of Biochemistry, School of Biological Sciences, University of Bath, Claverton Down, Bath, Avon, England

Received July 7, 1980

A method is described for analyzing the extent of mono- and oligo-ADP-ribosylation of specific nuclear proteins directly from the protein bands on polyacrylamide gels. Ethylamine treatment of the gel slices containing the [^{3}H]ADP-ribosylated protein results in the release of the protein associated [^{3}H]mono- and oligo-ADP-ribose which are recovered following lyophilization of the ethylamine extract. Subsequent aminoethyl cellulose chromatography of this released material permits a quantitative separation of mono-ADP-ribose and its derivatives from oligo-ADP-ribose as evidenced by thin-layer chromatographic analysis of the separated fractions. This methodology and the determination of the extent of mono- and oligo-ADP-ribosylation is demonstrated here using [^{3}H]ADP-ribosylated H1 synthesized in pig thymus nuclei incubated in the presence of [^{3}H]NAD. The method is shown to be applicable to both sodium dodecyl sulfate and acid-urea polyacrylamide gels. Furthermore, by performing such analyses on purified and crude H1 preparations the method is shown to allow accurate determinations of the extent of mono- and oligo-ADP-ribosylation without having to first purify the acceptor protein to homogeneity.

ADP ribosylation of proteins has been observed to occur in the nuclei of all eukaryotes so far studied (1–3). This covalent modification of nuclear proteins is catalyzed by a chromatin-bound enzyme, poly(ADP-ribose)[1] synthetase, which catalyzes the incorporation of the ADP-ribose moiety of NAD into mono- and poly-ADP-ribosylated nuclear proteins (1–3). Despite the implication of ADP ribosylation in DNA replication and repair, transcription, differentiation, and cell growth and proliferation (for a recent review see Ref. (1)) its precise role remains unknown. One necessary approach is to characterize the ADP ribosylation of specific proteins. To date the most commonly used approach has been to isolate and study those proteins for which relatively quick and easy methods of purification are available, e.g. the histones. Even though much information has been obtained on the ADP ribosylation of histones analysis of other specific acceptor proteins has proved more difficult (1). A more recent approach has been to separate ADP-ribosylated proteins from unmodified proteins using boronate affinity chromatography (4–7). Even using this latter method, analysis of individual ADP-ribosylated proteins has only been achieved when used in conjunction with other specific protein, purification steps as is the case for A24 protein (5), and the histones (6). Clearly methodology needs to be developed which will enable the ADP ribosylation of individual proteins to be analyzed rapidly without the

[1] Abbreviations used: ADP, adenosine diphosphate; ADP-ribose, adenosine diphosphate-ribose; AMP, adenosine monophosphate; HMG proteins, high-mobility group proteins; PCA, perchloric acid; PPO, 2,5-diphenyloxazole; PEI-cellulose, polyethylene imine-cellulose; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

0003-2697/80/000000-00$00.00/0

need to go through the lengthy process of purifying to homogeneity each possible acceptor protein. It is such methodology which we wish to report in this communication. We have developed a procedure which permits the analysis of the ADP ribosylation of individual proteins directly from protein bands on polyacrylamide gels. The efficiency and reliability of the method is demonstrated using [^{3}H]ADP-ribosylated histone H1 isolated in a completely pure, and a crude, form from pig thymus nuclei which have been incubated with [^{3}H]NAD.

MATERIALS AND METHODS

Adenosine-[^{3}H]NAD (1 mCi/ml, 20 mCi/μmol) was synthesized from [^{3}H]ATP (Radiochemical Centre, Amersham) by the method of Ohtsu and Nishizuka (8).

Isolation of pig thymus nuclei. Nuclei were isolated as described by Khan and Shall (9).

Synthesis and isolation of [^{3}H]ADP-ribosylated H1. Isolated nuclei (approx 8 mg DNA were incubated for 5 min at 25°C with 200 μCi [^{3}H]NAD in 3 ml of reaction mixture which contained 2 mM dithiothreitol, 10 mM $MgCl_2$, and 50 mM triethanolamine/HCl, pH 8.2. Then 0.75 ml 2 N H_2SO_4 was added (final concentration of 0.4 N) and the histones were extracted as previously described (10). Histone H1 was isolated from the total histone preparation by extraction with 5% (v/v) PCA (11) and purified to homogeneity by precipitation of the H1 from the PCA with 3.5 vol of acetone and 0.03 vol of concentrated HCl (12).

Analysis of incorporated [^{3}H]NAD. The extent of [^{3}H]ADP ribosylation of the histones and H1 was determined using the filter disc assay as previously described except that all the TCA washes were done with 20% (w/v) TCA (13).

Polyacrylamide gel electrophoresis of total histones and H1. Electrophoresis was performed using the 6.25 M urea–0.9 N acetic acid system of Panyim and Chalkley (14). SDS–polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (15). Gels were sliced and analyzed for radioactivity using the one-step procedure of Aloyo (16).

Extraction and separation of [^{3}H]mono- and oligo-ADP-ribose from gels. Gel slices (1.5 mm) containing the [^{3}H]ADP-ribosylated H1 were placed in fixing solution (45.4% (v/v) methanol–9.2% (v/v) acetic acid) in a tightly capped bottle and left overnight at room temperature. The fixing solution was then decanted and the gels were quickly rinsed with distilled water before incubating them with 1 M ethylamine (1 ml/gel slice) for 7 h at 37°C. (During both the fixing of the gels and the incubation with ethylamine the gels were agitated with the aid of a magnetic stirrer bar.) The ethylamine, which extracts mono-ADP-ribose, its derivatives and oligo-ADP-ribose, was then removed and lyophilized. The sample was dissolved in 6 N acetic acid and any fragments of gels present were removed by centrifugation for 10 min at 10,000*g*. (Alternatively, the sample may be centrifuged before the lyophilization step.) The 6 N acetic acid sample was then applied to an aminoethyl cellulose column (15 × 10 mm), eluted with 1-ml volumes of 6 N acetic acid and 1-ml fractions were collected until no more radioactivity eluted from the column. The elution was continued with 1-ml volumes of distilled water (approx 6) and finally with 1-ml volumes of 1 M ethylamine.

Analysis of aminoethyl cellulose fractions. In order to check that mono-ADP-ribose and its derivatives were completely separated from oligo-ADP-ribose, by aminoethyl cellulose chromatography the 6 N acetic acid and 1 M ethylamine fractions were lyophilized and analyzed by PEI-cellulose thin-layer chromatography as previously described (17). Following chromatography each sample track was cut into 0.5-cm-wide strips which were placed into

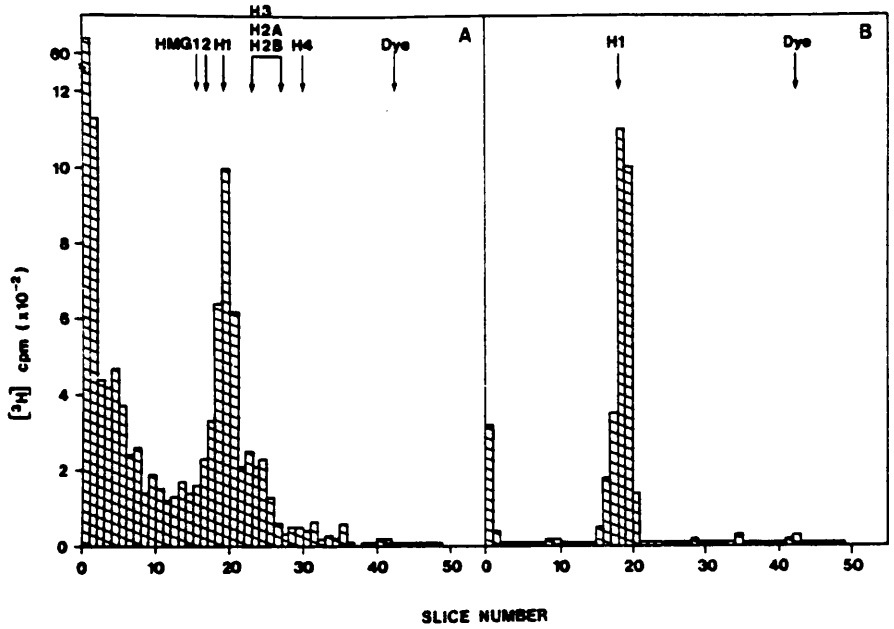


FIG. 1. Electrophoresis of [³H]ADP-ribosylated total histones and purified histone H1 on acid-urea polyacrylamide gels. Nuclei were incubated with [³H]NAD, total histones, and purified H1 isolated and aliquots of each were analyzed by gel electrophoresis. (A) 50 μg crude total histones (approx 15,000 cpm); (B) 10 μg pure histone H1 (approx 3000 cpm).The arrows indicate the position of the proteins seen on duplicate stained gels.

capped scintillation vials containing 1 ml 5% (v/v) PCA and the vials were heated at 70°C for 15 min. After cooling the radioactivity in each strip was determined by adding 10 ml of scintillation fluid (0.5% (w/v) PPO–70% (v/v) toluene–30% (v/v) Triton X-100) and counting in a Packard Tri Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

In order to develop new techniques which will lead to a method for analyzing the ADP ribosylation of specific proteins on gels it is necessary to test possible approaches using "model proteins." The requirements of such a protein are that it is ADP ribosylated when nuclei are incubated with [³H]NAD, that it is easily and quickly isolated both in a semipure and pure form, and finally, that it is readily resolved and identified on polyacrylamide gels. Such requirements are fulfilled by histone H1 which has been shown to be ADP ribosylated in several tissues (see Ref. (1)). It is readily extracted from nuclei with 0.4 N H_2SO_4 in a semipure form along with the other histones (18) and may be further separated and purified by PCA extraction and acetone/HCl precipitation (12). Indeed, when pig thymus nuclei are incubated with [³H]NAD and the 0.4 N H_2SO_4 extract is analyzed by acid–urea polyacrylamide gel electrophoresis it is seen that histone H1 appears to be the major protein ADP ribosylated with the H1-associated radioactivity accounting for approximately 20% of the total in the preparation (Fig. 1A). Acid–urea polyacrylamide gel analysis of H1 which was purified to homogeneity, as evidenced by a single band on SDS–gels and acid–urea gels (data not shown), shows that greater than 90% of the radioactivity in the H1 preparation is asso-

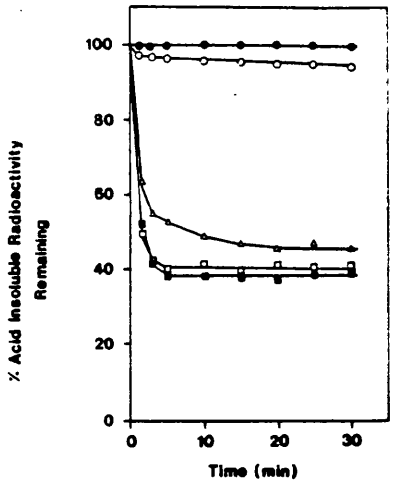


FIG. 2. Stability of [^{3}H]ADP-ribose H1 linkages. Purified histone H1 (60μg, 18,000 cpm) was incubated under various conditions in a final volume of 0.2 ml at 37°C. At different times 20-μl aliquots were taken and the acid-insoluble radioactivity remaining determined using the filter disc assay as described under Materials and Methods. (●), 0.9 N acetic acid; (○) 0.1 M sodium phosphate, pH 7; (△), 0.4 N NH_4OH, pH 7; (□) 1 M ethylamine; (■) 0.1 M NaOH.

ciated with H1 (Fig. 1B). These results clearly indicate H1 to be an ideal "model protein" and the methodology presented in this communication has been developed using such H1 preparations as seen in Fig. 1.

It is essential that the [^{3}H]ADP-ribose can be released from the modified H1 on gels in order to be able to characterize the radioactivity. Basic conditions are known to quantitatively cleave all mono-ADP-ribose and oligo-ADP-ribose–protein linkages (19) and so treatment of gel slices containing [^{3}H]ADP-ribosylated H1 under such conditions should result in release of the radioactivity. In order to simplify processing of the sample following incubation of the gel slices with base, ethylamine was chosen instead of the commonly used NaOH (19). Ethylamine is extremely volatile and hence is easily removed prior to further analysis. The H1–ADP-ribose linkages appear to be equally sensitive to 1 M ethylamine and 0.1 N NaOH (Fig. 2) suggesting that 1M ethylamine cleaves all linkages as does NaOH (19). Analysis of H1 which had been pretreated with 1M ethylamine for 30 minutes at 37°C on acid-urea polyacrylamide gels confirmed that 1M ethylamine does quantitatively cleave all H1-ADP-ribose linkages since, unlike the control (Fig. 1B) no radioactivity was observed electrophoresing on the gel with H1 (data not shown). (The stability of the H1-ADP-ribose linkages towards 0.9N acetic acid and 0.1M sodium phosphate pH 7, which act as controls for the stability on acid-urea and SDS gels respectively, is also seen in Fig. 2. There is no detectable cleavage in acetic acid although some small loss is observed at pH 7 but not sufficient to invalidate analysis by SDS–gel electrophoresis at this pH. Incubation with neutral hydroxylamine, which has been shown to cleave only a proportion of mono- and oligo-ADP-ribose–protein bands (19), results in less cleavage than NaOH and ethylamine which indicates some hydroxylamine-resistant linkages on H1 (Fig. 2). Such ADP-ribose linkages have been analyzed and the results are discussed later.

Thus, having established that ethylamine does cleave all the [^{3}H]ADP-ribose-H1 bands the kinetics of the release of such radioactivity from H1 on gels was studied in order to determine the conditions necessary for recovery of the counts. H1 preparations containing [^{3}H]ADP-ribosylated H1 were electrophoresed on SDS- and on acid–urea polyacrylamide gels and following slicing of the gels those slices containing [^{3}H]ADP-ribosylated H1 were fixed as described under Materials and Methods.[2] Fixing of the gels is important because it

[2] Under standard electrophoretic conditions as used in this present study the slices containing the [^{3}H]-ADP-ribosylated H1 are Nos. 16–21 for acid–urea gels when the marker dye electrophoreses 7.3 cm (slice 43) and Nos. 10–15 for SDS–gels when the marker dye electrophoreses 7.5 cm.

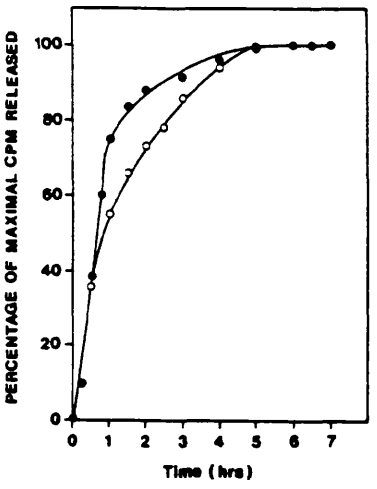


FIG. 3. Time course of release of [^{3}H]ADP-ribose from acrylamide gel slices by ethylamine. Gel slices containing a total of 24,000 cpm of [^{3}H]ADP-ribosylated histone H1 were fixed and treated with 40 ml 1 M ethylamine as described under Materials and Methods. At various times of incubation with ethylamine 1-ml aliquots were taken and the released radioactivity was determined by counting in PPO–toluene–X-100 scintillation fluid. The counts per minute released at 7 h (approx 600 cpm) were taken as the maximum and the percentage of maximum counts per minute released was calculated for each time (●), SDS gels; (○) acid–urea gels.

facilitates the removal of compounds such as SDS and phosphate buffer which may interfere with the subsequent analysis of the released radioactivity by aminoethyl cellulose chromatography. The gel slices were then incubated with 1 ml/slice ethylamine[3] at 37°C and at various time aliquots taken and the counts released were determined. The results are seen in Fig. 3 and it is clear that by 6 h a quantitative recovery of released radioactivity is obtained for samples electrophoresed on SDS–gels and on acid–urea gels. The released radioactivity should consist of oligo-ADP-ribose and the base products of mono-ADP-ribose, namely, 5′-AMP and "*X*" an ADP-like compound (20). Separation and determination of the radioactivity in the oligo- and mono-ADP-ribose fractions would permit conclusions to be made regarding the extent of mono- and oligo-ADP ribosylation of the acceptor protein. Separation of mono-ADP-ribose and its based-derived products from oligo-ADP-ribose have been extensively studied in this laboratory (Purnell and Whish, unpublished data) and the procedure adopted here results from such studies. Histones were treated with 1 M ethylamine and lyophilized prior to solubilization in 6 N acetic acid and aminoethyl cellulose chromatography performed as described under Materials and Methods. A quantitative recovery of radioactivity was obtained which eluted in two fractions—a 6 N acetic acid and a 1 M ethylamine fraction (Fig. 4). The two fractions were lyophilized

[3] The gel slices swell in ethylamine and absorb approximately four times their own volume of solution (100 μl). One milliliter ethylamine/gel slice results, therefore, in only a 10% loss which can be further reduced by increasing the volume of ethylamine used.

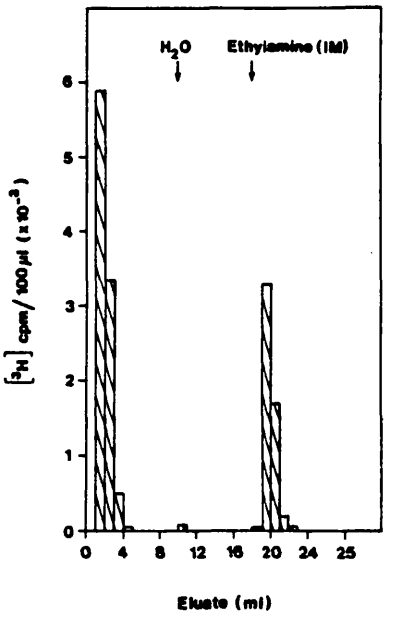


FIG. 4. Aminoethyl cellulose chromatography of [^{3}H]mono- and oligo-ADP-ribose released from total histones. [^{3}H]ADP-ribosylated total histones (150,000 cpm and 0.5 mg protein) were incubated with 1 M ethylamine at 37°C for 7 h before lyophilizing and chromatography as described under Materials and Methods.

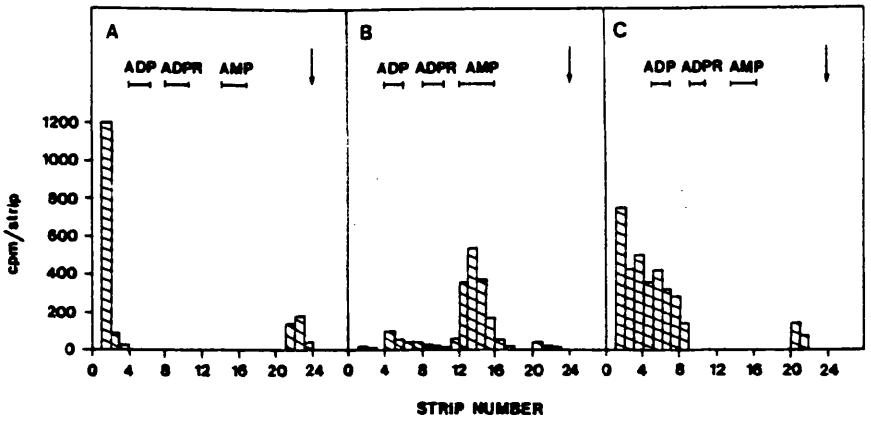


FIG. 5. PEI-cellulose chromatography of the ethylamine-released products from [^{3}H]ADP-ribosylated total histones. The resolved acetic acid and ethylamine fractions obtained after aminoethyl cellulose chromatography of ethylamine-treated [^{3}H]ADP-ribosylated total histones (Fig. 3) were lyophilized and analyzed by PEI-cellulose chromatography as described under Materials and Methods. (A) untreated and unfractionated histones, (B) acetic acid fraction, (C) ethylamine fraction. The arrow indicates the position of the solvent front; ADP, ADP-ribose, and AMP were included as markers.

and analyzed by PEI-cellulose thin-layer chromatography in order to check the separation afforded by the aminoethyl cellulose. The results are shown in Fig. 5 and it can be seen that in the original untreated H1–ADP-ribose preparation the counts remain on the origin because the ADP-ribose is bound to the H1 (Fig. 5A). The 6 N acetic acid fraction is seen to consist mostly of 5′-AMP with traces of ADP and ADP-ribose with no counts on the origin or between the origin and ADP (Fig. 5B). Such are the expected products of base-hydrolyzed mono-ADP-ribose (20) and the results demonstrate that the N acetic acid fraction from the aminoethyl cellulose column contains no oligo-ADP-ribose and only mono-ADP-ribose and its derivatives. Analysis of the 1 M ethylamine fraction shows the radioactivity to be on the origin and between the origin and ADP-ribose with no counts chromatographing with ADP-ribose or 5′-AMP (Fig. 5C). Such a result demonstrates the absence of any mono-ADP-ribose or its derivatives in the 1 M ethylamine fraction and the presence of only oligo-ADP-ribose. Thus aminoethyl cellulose chromatography clearly permits separation of mono-ADP-ribose and its derivatives from oligo-ADP-ribose. Therefore the extent of mono- and oligo-ADP-ribosylation of specific proteins can readily be determined by processing gel slices containing the [^{3}H]ADP-ribosylated protein with ethylamine prior to separation of the released material by aminoethyl cellulose chromatography. Such determinations have been made on purified H1 which has been electrophoresed on SDS– and acid–urea polyacrylamide gels and the values obtained compared with the value from purified H1 obtained directly without electrophoresis on gels. The results are given in Table 1 (*d–f*) and it can be seen that the three values are very close and show that H1 is approximately 40% mono-ADP ribosylated and 60% oligo-ADP isolated. This demonstrates that the analysis of ADP-ribosylated H1 on polyacrylamide gels produces the same values as one would obtain using H1 directly and rules out the possibility that interference by the gels may lead to erroneous results. The same value of 40% mono-ADP ribosylation and 60% oligo-ADP ribosylation is also seen when total histones are electrophoresed on acid–

urea gels and the [^{3}H]ADP-ribosylated H1-containing slices (Nos. 16–21) is analyzed (Table 1 (*g*)). This result shows that an accurate specific analysis of the ADP-ribosylation of H1 can be performed on a crude sample of H1, in this case a total histone preparation, without the need to first purify the H1. This is clearly a great advantage since one removes not only the need to purify the H1 but also the losses of radioactivity and protein likely to occur during such a purification.

It has been shown that at least two types of bands exist between ADP-ribose and protein one of which is cleaved both by NaOH and neutral hydroxylamine and one which is cleaved only by NaOH and not by neutral hydroxylamine (19). Thus, using the methodology described here it should be possible to obtain information concerning the hydroxylamine resistance of both the mono- and oligo-ADP-ribose which is attached to protein. We have performed such an analysis using H1. Treatment of histone H1 or total histones with neutral hydroxylamine and subsequent acid–urea gel electrophoresis show that approximately 27% of the H1-associated ADP-ribose is resistant (data not shown). Cleavage of this resistant fraction with ethylamine and aminoethyl cellulose chromatography indicates that approximately 20% of this resistant fraction is mono-ADP-ribose and 80% is oligo-ADP-ribose (Table 1 (*h*)). This means that 5.4% of the total H1-associated radioactivity is hydroxylamine-resistant monomer and 21.6% of the total is hydroxylamine-resistant oligo-ADP-ribose. Since 40% of all H1 associated ADP-ribose is monomer this means that 13.5% (5.4 out of 40) of all H1-associated mono-ADP-ribose is hydroxylamine resistant.

Since there are no other data available on the extent of mono ADP ribosylation and oligo-ADP ribosylation of H1 *in vitro* we have no true check on the accuracy of

TABLE 1

EXTENT OF MONO- AND OLIGO-ADP-RIBOSYLATION OF HISTONE H1[a]

Sample	No. of determinations	Monomer		Oligomer	
		cpm[b]	%[c]	cpm[b]	%[c]
Pure H1[d]	2	11,320 ± 205	39.86	17,078 ± 237	60.14
Pure H1–acid–urea gels[e]	3	4,031 ± 135	40.85	5,835 ± 47	59.15
Pure H1–SDS–gels[f]	3	1,840 ± 68	38.79	2,904 ± 72	61.21
H1 from total histones on gels, control[g]	3	9,423 ± 208	39.27	14,575 ± 217	60.73
H1 from total histones on gels, + NH_2OH[h]	3	1,458 ± 47	19.38	6,064 ± 119	80.62

[a] Various [^{3}H]ADP-ribosylated H1 preparations were treated with ethylamine and the amount of released mono- and oligo-ADP-ribose, which eluted from an aminoethyl cellulose column with 6 N acetic acid and 1 M ethylamine, respectively, was determined (see Materials and Methods).

[b] Mean values ± standard error are given.

[c] Values are expressed as a percentage of the combined monomer and oligomer which is taken as 100%.

[d] Purified H1 was treated directly with ethylamine.

[e] Purified H1 was electrophoresed on acid–urea gels and the H1-containing gel slices were treated with ethylamine.

[f] Purified H1 was electrophoresed on SDS–gels and the H1-containing gel slices were treated with ethylamine.

[g] Total histones were electrophoresed on acid–urea gels and the H1-containing gel slices treated with ethylamine.

[h] Total histones which had been treated with 0.4 M NH_2OH pH 7 for 30 min at 37°C were electrophoresed on acid–urea gels and the H1-containing gel slices were treated with ethylamine.

TABLE 2

AVERAGE CHAIN LENGTH OF H1-ASSOCIATED ADP-RIBOSE[a]

Sample	Average chain length: Total ADP-ribose[b]	Average chain length: Oligo-ADP-ribose[c]
Pure H1[d]	1.62	3.10
Pure H1–acid–urea gels[e]	1.57	2.98
Pure H1–SDS–gels[f]	1.64	3.05
H1 from total histones on gels, control[g]	1.48	3.26
H1 from total histones on gels, + NH_2OH[h]	2.21	3.23

[a] Various [^{3}H]ADP-ribosylated H1 preparations were treated with ethylamine and the average chain length of the released material calculated (22) following treatment with snake venom phosphodiesterase and thin-layer chromatography (17).

[b] Total released ADP-ribose (monomer and oligomer).

[c] Oligo-ADP-ribose released and eluted free of any mono-ADP-ribose, from aminoethyl cellulose with ethylamine.

[d–h] As for footnote to Table 1.

the values reported here. However, the data presented in Fig. 2 allow one to set a limit for the values one should expect which acts as a check on the accuracy of the presently described method. In the case of hydroxylamine cleavage approximately 7% of the total H1–ADP-ribose bands are resistant when compared with NaOH treatment (Fig. 2). Since the total hydroxylamine resistance is 27% (see above) this means that 20% of the resistant material is oligomer which is long enough to be acid precipitable even when cleaved by NaOH. This produces a ratio for acid soluble:acid insoluble of 7:20 (approx 0.33). Clearly some of the 7% radioactivity which is rendered acid soluble by NaOH should be of chain lengths greater than mono-ADP-ribose and hence will be retained by aminoethyl cellulose and elute with 1 M ethylamine in the oligo-ADP-ribose fraction. Thus one should expect a ratio of mono-ADP-ribose:oligo ADP-ribose somewhat less than 0.33 using the present gel analysis methodology. The results in Table 1 (*h*) show a ratio of 19.38: 80.62 = 0.24. Quantitative cleavage of H1–ADP-ribose conjugates with ethylamine results in a loss of 60% of the original acid-insoluble radioactivity (Fig. 2). Since some of this material should be of chain length greater than mono-ADP-ribose this value of 60% is the maximum value one should obtain for mono-ADP-ribose and 40% is the minimum value one should obtain for oligo ADP-ribose. The approximate values obtained in this present study are 40% mono-ADP-ribose and 60% oligo-ADP-ribose (Table 1). Thus the values obtained using the presently described methodology are in good agreement with those predicted from the filter disc assay results shown in Fig. 2.

The methodology described here also permits determinations to be made of the average chain length of the oligomeric ADP-ribose which is attached to the acceptor proteins. We have performed such analyses on ADP-ribosylated histone H1 preparations which have been processed in different ways and the results are given in Table 2. It is clear that there is good agreement between the value obtained from purified H1 (Table 2 (*d*)) and the values obtained from purified and crude H1 electrophoresed on polyacrylamide gels (Table 2 (*e*–g)). Such results strengthen our conclusions derived from the data in Table 1 that an accurate analysis of the ADP ribosylation of H1 can be performed on a crude sample of H1 without the need to first purify H1. It can also be seen from Table 2 that determination of the average chain length of total ADP-ribose produces a lower value than that of the separated oligo-ADP-ribose fraction. Such values for oligomer are in good agreement with the values one should expect from the known distribution of mono- and oligo-ADP-ribose on H1 (Table 1) and the average chain length of the total ADP-ribose associated with H1 (Table 2). Finally it can be seen from the

results in Table 2 that the average chain length of the oligo-ADP-ribose which is attached to H1 via an Nh_2OH-resistant linkage (Table 2 (*h*)) is very similiar to the average chain length of the total oligo-ADP-ribose.

Such results clearly demonstrate the potential of this method in allowing not only an analysis of hydroxylamine resistant and sensitive mono- and oligo-ADP-ribose but also a determination of the average chain length of such fractions associated with specific proteins. The method is applicable to any protein because the method relies not on any specific property of the protein but on the quantitative hydrolysis of the ADP-ribose protein linkage under basic conditions. Thus using this method it should be possible to analyze whole gel profiles of ADP-ribosylated proteins and to characterize specific protein bands of interest as a function of whatever cell parameter one is investigating. We are presently applying the methodology described here to an analysis of [^{3}H]ADP-ribosylated proteins synthesized *in vivo* in mouse L1210 cells grown in the presence of [^{3}H]adenosine and isolated by CsCl equilibrium density gradient centrifugation as previously described (21). Preliminary studies employing aminoethyl cellulose chromatography to separate *in vivo* protein-associated mono- and oligo-ADP-ribose isolated from L1210 cells indicate that 75% of the *in vivo* protein-bound ADP-ribose is monomer with the remaining 25% being oligomer of average chain length approximately 2.9 (23).

ACKNOWLEDGMENTS

We thank the Science Research Council (support to MRP, PRS, and WJDW), the Medical Research Council (support to WJDW) and Miles Laboratories (support to MRP and WJDW).

REFERENCES

1. Purnell, M. R., Stone, P. R., and Whish, W. J. D. (1980) *Biochem. Soc. Trans.* **8**, 215–227.
2. Hilz, H., and Stone, P. R. (1976) *Rev. Physiol. Biochem. Pharmacol.* **76**, 1–58.
3. Hayaishi, O., and Ueda, K. (1977) *Annu. Rev. Biochem.* **46**, 95–116.
4. Okayama, H., Ueda, K., and Hayaishi, O. (1978) *Proc. Nat. Acad. Sci. USA* **75**, 1111–1115.
5. Okayama, H., and Hayaishi, O. (1978) *Biochem. Biophys. Res. Commun.* **84**, 755–762.
6. Adamietz, P., Klapproth, K., and Hilz, H. (1979) *Biochem. Biophys. Res. Commun.* **91**, 1232–1238.
7. Minaga, T., Romaschin, A. D., Kirsten, E., and Kun, E. (1979) *J. Biol. Chem.* **254**, 9663–9668.
8. Ueda, K., and Yamamura, H. (1971) *in* Methods in Enzymology (McCormick, D. B., Wright, L. D., eds.), Vol. 18B, pp. 60–67, Academic Press, New York.
9. Khan, M. G., and Shall, S. (1976) *Biochem. Soc. Trans.* **4**, 778–781.
10. Whitby, A. J., Stone, P. R., and Whish, W. J. D. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1295–1304.
11. Johns, E. W. (1964) *Biochem. J.* **92**, 55–59.
12. Goodwin, G. H., and Johns, E. W. (1977) *in* Methods in Cell Biology (Prescott, D. M., ed.), Vol. 26, pp. 257–267, Academic Press, New York.
13. Purnell, M. R., and Whish, W. J. D. (1980) *Biochem. J.* **185**, 775–777.
14. Panyim, S., and Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346.
15. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
16. Aloya, U. J. (1979) *Anal. Biochem.* **99**, 161–164.
17. Stone, P. R., Whish, W. J. D., and Shall, S. (1973) *FEBS Lett.* **36**, 334–337.
18. Johns, E. W. (1971) *in* Histones and Nucleohistones (Phillips, D.M.P., ed.), pp. 1–45, Plenum Press, New York.
19. Adamietz, P., and Hilz, H. (1976) *Hoppe Seyler's Z. Phys. Chem.* **357**, 527–534.
20. Goebel, M., Stone, P. R., Lengyel, H., and Hilz, H. (1977) *Hoppe Seyler's Z. Phys. Chem.* **358**, 13–21.
21. Purnell, M. R., Stone, P. R., Surowy, C., and Whish, W. J. D. (1980) *in* Proceedings of Conference on Novel ADP-ribosylation of Regulatory Enzymes and Proteins (Sugimura, T., and Smulson, M., eds.), Elsevier, North-Holland, Amsterdam.
22. Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M., and Hayaishi, O. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 781–786.
23. Purnell, M. R., Stone, P. R., Surowy, C. S., and Whish, W. J. D. (1981) *Biochem. Soc. Trans.*, in press.